JCI The Journal of Clinical Investigation

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J Clin Invest. 2010;120(6):2109-2118. https://doi.org/10.1172/JCI39964.

Research Article **Hematology**

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Mcl1 haploinsufficiency protects mice from *Myc*-induced acute myeloid leukemia

Zhifu Xiang,[1] Hui Luo,[1] Jacqueline E. Payton,[2] Jennifer Cain,[3] Timothy J. Ley,[1] Joseph T. Opferman,[4] and Michael H. Tomasson[1]

[1]Department of Medicine, Division of Oncology, and [2]Department of Pathology and Immunology, Siteman Cancer Center, Washington University School of Medicine, St. Louis, Missouri, USA. [3]OncoMed Pharmaceutical Inc., Redwood City, California, USA. [4]Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, Tennessee, USA.

Antiapoptotic BCL2 family members have been implicated in the pathogenesis of acute myelogenous leukemia (AML), but the functional significance and relative importance of individual proteins (e.g., BCL2, BCL-XL, and myeloid cell leukemia 1 [MCL1]) remain poorly understood. Here, we examined the expression of *BCL2*, *BCL-XL*, and *MCL1* in primary human hematopoietic subsets and leukemic blasts from AML patients and found that *MCL1* transcripts were consistently expressed at high levels in all samples tested. Consistent with this, Mcl1 protein was also highly expressed in myeloid leukemic blasts in a mouse *Myc*-induced model of AML. We used this model to test the hypothesis that Mcl1 facilitates AML development by allowing myeloid progenitor cells to evade *Myc*-induced cell death. Indeed, activation of *Myc* for 7 days in vivo substantially increased myeloid lineage cell numbers, whereas hematopoietic stem, progenitor, and B-lineage cells were depleted. Furthermore, *Mcl1* haploinsufficiency abrogated AML development. In addition, deletion of a single allele of *Mcl1* from fully transformed AML cells substantially prolonged the survival of transplanted mice. Conversely, the rapid lethality of disease was restored by coexpression of *Bcl2* and *Myc* in *Mcl1*-haploinsufficient cells. Together, these data demonstrate a critical and dose-dependent role for Mcl1 in AML pathogenesis in mice and suggest that MCL1 may be a promising therapeutic target in patients with de novo AML.

Introduction

Acute myeloid leukemia (AML) affects 10 in 100,000 individuals in the United States and is fatal without treatment. Standard chemotherapy consists of a combination of anthracycline and cytarabine and yields a 5-year survival rate of 20%–30% (1). Despite decades of clinical research, the treatment for AML has remained essentially unchanged for 30 years (1). Defects in apoptosis pathways are common and perhaps essential for tumorigenesis and maintenance (2). Overexpressed antiapoptosis BCL2 family members render tumor cells more resistant to conventional chemotherapy (3). On the other hand, all tumor cells retain their normal cell death machinery (4). Thus, therapeutic strategies targeting overexpressed *BCL2* or other BCL2 family members are attractive and emerging from a better understanding of molecular mechanisms underlying apoptosis regulation and of dysregulated pathways for cancer. Indeed, the identification of antiapoptosis BCL2 family members and development of BH3 mimetic small-molecular inhibitors has led to enthusiasm among oncologists (3), but the finding that these inhibitors may have differential specificity among BCL2 family members has highlighted the importance of characterizing the role of individual family members in specific cancer types (5).

The prototypic oncogene *Myc* encodes a helix-loop-helix leucine zipper transcription factor with diverse functions, including cell cycle progression, metabolism, angiogenesis, differentiation, and apoptosis (6–8). Dysregulated *Myc* has been found in a large fraction of human cancers, and extensive studies have shed light on the mechanisms by which *Myc* overexpression promotes tumorigenesis (9–12). Retrovirally mediated overexpression of *C-Myc* (13) or *N-Myc* (14) in mouse bone marrow cells rapidly induces an oligoclonal AML-like disease characterized by splenomegaly; accumulation of immature myeloid cells in bone marrow, spleen, thymus, and lymph nodes; hind limb paralysis; and death within 6 weeks. *Myc* induces AML in both C57BL/6 and Balb/c strains of mice with similar latency, and the leukemia is readily transplantable into secondary recipients (13). Human and mouse *Myc* genes, and both p62 and p67 *Myc* isoforms, all induce AML if expressed at sufficiently high levels (H. Yung and M.H. Tomasson, unpublished observations). Expression of *Myc* via retroviral transduction-transplantation using *Ink4a*$^{-/-}$ donor cells, or coexpression of *Myc* with *Bcl2*, cooperatively induces B cell leukemia/lymphoma (13), consistent with the data generated using B cell–specific transgenic mice (9). However, *Ink4a* status and *Bcl2* coexpression did not affect AML development in this model, and, in contrast to lymphomas developing in *Myc* transgenic mice, we found that AML tumors harbor neither karyotypically detectable chromosomal abnormalities nor mutations in the *Ink4a-p53* tumor suppressor pathway (13). Taken together, these data suggest that myeloid progenitor cells expressing *Myc* in bone marrow transplantation-transduction assays possess an intrinsic mechanism of resistance to *Myc*-induced apoptosis.

We sought to evaluate the role of BCL2 family members in the pathogenesis of AML using a *Myc*-induced mouse AML model. We hypothesized that retroviral expression of *Myc* rapidly induces myeloid lineage leukemia, at least in part, by cooperating with intrinsic apoptosis resistance factors of bone marrow progenitor subpopulations. Here, we sought to identify factors required for AML development by examining human AML samples and fractionated subpopulations of bone marrow cells from leukemic mice by Western blot analysis of candidate apoptosis proteins. We identified several candidate apoptosis gene products that were differentially expressed between myeloid and lymphoid hematopoietic cells in mice, and focused our validation efforts on the

Conflict of interest: The authors have declared that no conflict of interest exists.
Citation for this article: *J Clin Invest.* 2010;120(6):2109–2118. doi:10.1172/JCI39964.

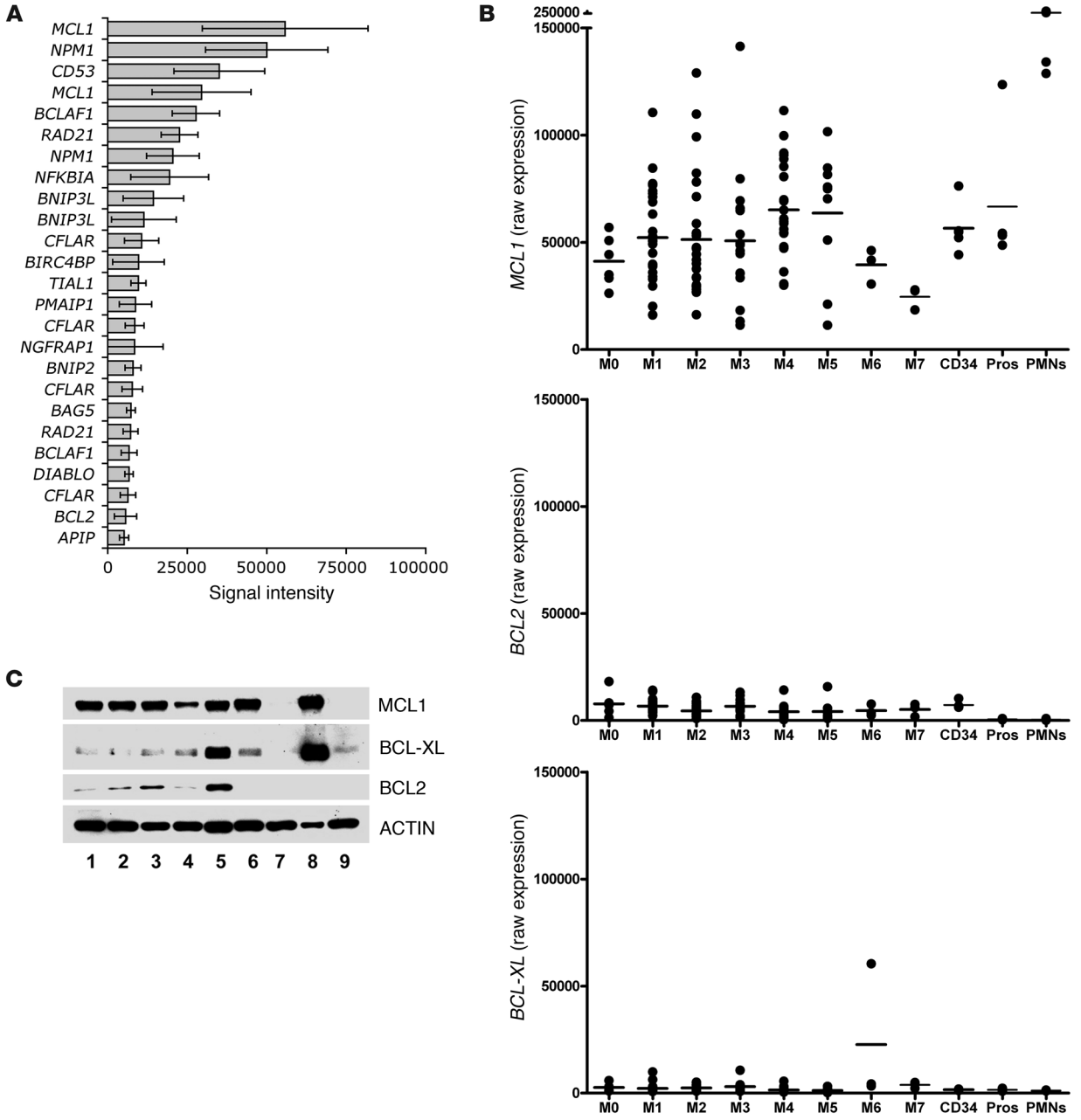

Figure 1

MCL1 is the predominant BCL2 family member expressed in primary AML samples. (**A**) Gene expression profiles were analyzed in 111 de novo AML samples, and high *MCL1* expression was observed in all AML samples. Error bars represent mean ± SD. (**B**) *MCL1*, but not *BCL2* and *BCL-XL*, were highly expressed in all AML subtypes. Expression levels of *MCL1*, *BCL2*, and *BCL-XL* were analyzed in AML samples of different French, American, and British (FAB) classification subtypes (M0–M7) and in cells from normal donors (sorted $CD34^+$ cells, promyelocytes [Pro], and PMNs). Each data point represents 1 sample (either patient or sorted normal cells). (**C**) Elevated MCL1 expression was seen in almost all AML samples. Western blots of AML cells from newly diagnosed AML patients. Lanes 1–6, AML samples; lane 7, chronic myeloid leukemia sample; lane 8, K562 cell line; lane 9, normal human bone marrow mononuclear cell control.

BCL2 family member myeloid cell leukemia 1 (*Mcl1*), based on the high expression of *MCL1* in 100% of human de novo AML samples examined. We hypothesized that if *Mcl1* is critically important for myeloid leukemogenesis, then decreased *Mcl1* gene dosage might protect mice from *Myc*-induced AML. Indeed, we found that haploinsufficiency of *Mcl1* significantly protected mice from *Myc*-induced AML, and that Cre-lox–mediated deletion of a single *Mcl1* allele prolonged the survival of mice with fully established AML. The critical sensitivity of the AML disease phenotype to *Mcl1* gene dosage demonstrates that *Mcl1* plays a nonredundant role in AML induction in mice and suggests that *MCL1* may play a similar role in human AML pathogenesis.

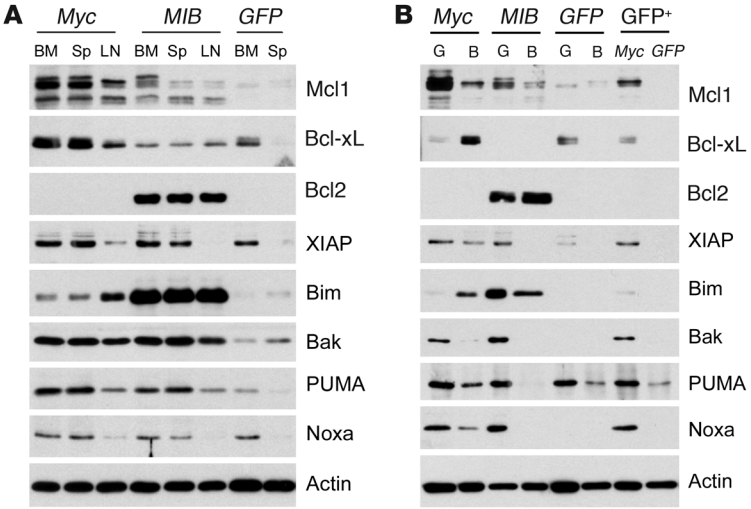

Figure 2
Elevated *Mcl1* expression in myeloid leukemia cells from *Myc*-induced AML mice. (**A**) Western blot of protein lysates prepared from unfractionated bone marrow, spleen (Sp), and lymph node cells isolated from leukemic mice transplanted with *MSCV-Myc* (*Myc*), *MSCV-Myc-Ires-Bcl2* (*MIB*), or vector alone (*GFP*). (**B**) Western blot of sorted Gr-1$^+$ myeloid (G) and B220$^+$ B-lymphoid (B) cells and cells sorted by GFP expression from *MSCV-Myc*– or *GFP*-transplanted mice. Cells were harvested from moribund leukemic mice, typically 4–6 weeks after transplantation, and from age-matched control mice. Representative blots from 3 independent experiments are shown.

Results

MCL1 expression is consistently high in primary human AML samples. *MCL1* was originally identified as a gene whose expression is induced during myeloid cell differentiation by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), and its antiapoptotic function may play a critical role in the survival of human leukemia cells (15, 16). We measured transcript levels using expression profiling from primary bone marrow samples derived from 111 de novo AML patients; of 174 candidate genes examined, *MCL1* transcripts were consistently high in all AML samples (Figure 1A) and all AML subtypes, whereas *BCL2* and *BCL-XL* expression levels were relatively low in AML samples (Figure 1B). In normal human bone marrow cells, *MCL1* transcripts increased with myeloid differentiation and remained high even in mature polymorphonuclear cells (PMNs); conversely, *BCL2* and *BCL-XL* transcripts either decreased or remained unchanged with normal hematopoietic differentiation (Figure 1B). We also examined the protein levels of MCL1, BCL2, and BCL-XL in bone marrow cells from the de novo AML patients and found that MCL1 proteins were expressed at high levels in almost all samples, whereas BCL2 and BCL-XL expression varied (Figure 1C). These data suggest that *MCL1* may play an important and previously unappreciated role in AML development.

Elevated Mcl1 expression in myeloid leukemia cells from Myc-induced AML mice. We then sought to test whether *Mcl1* expression is elevated in AML cells in mice from a murine AML model. We analyzed expression levels of *Mcl1* and other BCL2 family members in the *Myc*-induced AML mouse model (13). In this model, overexpression of *Myc* induces an exclusive AML phenotype in the absence of antiapoptotic mutations, whereas development of the lymphoid phenotype requires coexpression of *Myc* with *Bcl2*. We hypothesized that myeloid progenitors transformed by *Myc* must possess distinct machinery to overcome *Myc*-induced apoptosis and cause AML phenotype. To test our hypothesis, we used a transduction-transplantation approach, transplanting unfractionated murine bone marrow cells transduced with murine stem cell virus (MSCV) carrying *Myc* (*MSCV-Myc*) or *Myc-Ires-Bcl2* (*MSCV-Myc-Ires-Bcl2*; referred to herein as *MIB*) into leukemic mice. We then isolated mononuclear cells from the tissues of these mice and compared the expression of critical apoptosis proteins with that in control transplanted animals. Bone marrow and spleen cells from mice with *Myc*-induced AML (predominantly myeloid blasts; data not shown) exhibited increased levels of antiapoptotic proteins, especially Mcl1 and Bcl-xL (Figure 2A), but only moderately increased levels of Mcl1 and Bcl-xL were found in tissues of *MIB* mice (mixed myeloid and lymphoid blasts; data not shown). The lymph nodes from *Myc* recipients were phenotypically unaffected, whereas lymph nodes from *MIB* mice were infiltrated with lymphoid leukemia blasts (B220$^+$CD43$^+$ pre-B cells; data not shown), and only express modest levels of Mcl1. Furthermore, only the *MIB*-expressing mice had substantial levels of Bcl2 expression in comparison to *Myc* and vector-only recipients. Proapoptotic BH3-only family members PUMA, Bak, and Noxa were elevated in cells from *Myc* recipients (Figure 2A). Although Bim expression levels were elevated in *Myc* recipients, they were much higher in all tissues from *MIB* recipients (Figure 2A). These data suggest that the antiapoptotic proteins Mcl1 and Bcl-xL may be responsible for evading *Myc*-induced apoptosis and disease development.

We then used high-throughput flow cytometry to sort myeloid (Gr-1$^+$) and B lymphoid (B220$^+$) cells from the spleens of *Myc*, *MIB*, and vector-only recipient mice to assess whether the expression of apoptosis-related proteins is lineage specific. Interestingly, high Mcl1 expression was observed in sorted myeloid cells, but was relatively low in B-lineage cells (Figure 2B). Bcl-xL was highly expressed in *Myc*-transformed — but phenotypically normal — B cells from *Myc* mice, but not in immature B leukemic cells from *MIB* recipients (Figure 2B). Myeloid leukemic cells also expressed high levels of proapoptotic proteins Bak, Noxa, and PUMA, but not Bim (Figure 2B). Together, our results indicate that myeloid and lymphoid cell populations from normal and leukemic mice have distinct profiles of pro- and antiapoptotic proteins and that Mcl1 expression may enable myeloid progenitor cells to evade *Myc*-induced apoptosis.

Mcl1 is not a transcriptional target of Myc. Ectopic *Myc* expression can prime cells for programmed cell death, in part by downregulating *Bcl2* and *Bcl-xL* (17). We wondered whether *Myc* activation has the opposite effect on *Mcl1* expression. To address this question, NIH 3T3 cells and Ba/F3 cells were transduced with an inducible *Myc* allele, *MycER*, and Mcl1 expression levels were analyzed after *Myc* activation. To confirm activation of *Myc*, the

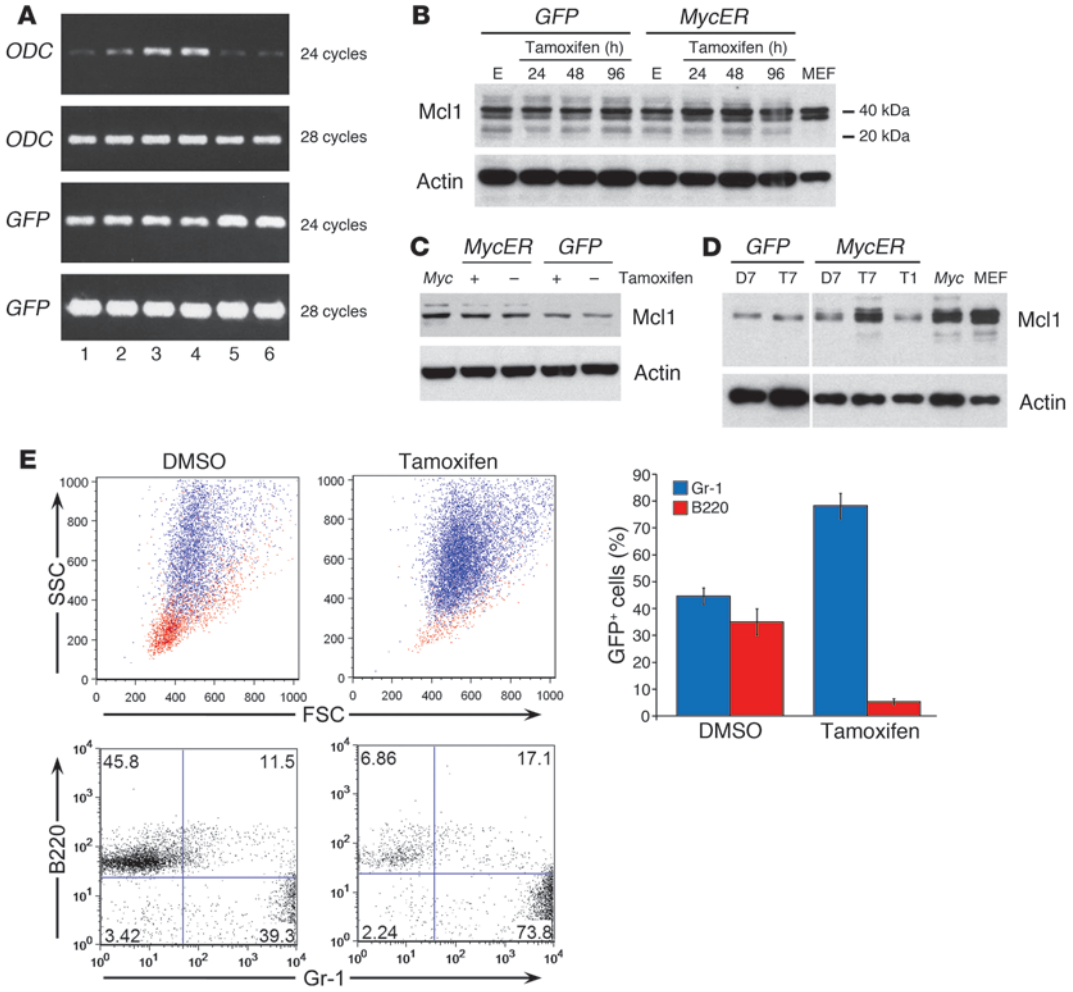

Figure 3

Myc activation does not directly alter *Mcl1* expression. (**A**) Elevated *Myc* target gene *ODC* expression upon *Myc* activation in 3T3 cells. RT-PCR was performed from RNA collected 0, 2, 4, and 6 hours after 4-OH tamoxifen treatment of *MycER*-transduced 3T3 cells (lanes 1–4, respectively) and *GFP*-transduced cells (0 and 6 hours; lanes 5 and 6, respectively). (**B**) Mcl1 expression was not changed in transduced BaF3 cells treated with ethanol (E) or with 4-OH tamoxifen dissolved in ethanol for the indicated times. SV40-transduced mouse embryonic fibroblasts (MEFs) were used as an Mcl1-positive control. (**C**) Mcl1 expression was not changed in transduced 3T3 cells treated for 24 hours with either 4-OH tamoxifen or ethanol. *Myc*-transduced 3T3 cells were used as a positive control. (**D**) Elevated Mcl1 expression in bone marrow cells after 7 days of *Myc* induction along with Gr-1$^+$ cell expansion in bone marrow. Protein lysates were prepared from the mice treated with DMSO for 7 days (D7) or tamoxifen for 1 or 7days (T1 and T7, respectively). SV40-transduced mouse embryonic fibroblasts and bone marrow cells of *Myc*-induced leukemic mice were used as positive controls. Lanes were run on the same gel but were noncontiguous (white lines). (**E**) Tamoxifen treatment resulted in relative depletion of B220$^+$ lymphoid cells with concurrent expansion of Gr-1$^+$ myeloid cells in bone marrow (blue, Gr-1$^+$; red, B220$^+$). FSC, forward scatter; SSC, side scatter. Cells were gated for GFP, and the percentages of Gr-1$^+$ and B220$^+$ events are shown at right. Data are mean ± SD.

expression level of its well-known target gene ornithine decarboxylase (*ODC*) was used as a readout of *Myc* activation (18). *ODC* transcript levels increased after 2 hours of treatment with 4-OH tamoxifen, confirming *Myc* activation (Figure 3A). However, Mcl1 expression was analyzed at different time points after 4-OH tamoxifen treatment, and no significant increases in Mcl1 protein levels were observed (Figure 3, B and C).

We also analyzed the Mcl1 expression levels in primary bone marrow cells after *Myc* induction. There were no changes in Mcl1 protein levels after 24 hours of in vivo *Myc* activation (Figure 3D). We found elevated Mcl1 levels in *MycER* bone marrow cells after 7 days of tamoxifen treatment; however, this increase was concomitant with expansion of the GFP$^+$GR-1$^+$ cell population (Figure 3E). Together, these data show that *Mcl1* is not a direct transcriptional target of *Myc*, but suggest that elevated Mcl1 levels in *Myc*-induced AML may be caused by indirect upregulation of *Mcl1*, or that *Myc* activation may provide a selective advantage to *Mcl1*-expressing cells.

We considered the possibility that one mechanism for high *Mcl1* expression in *MSCV-Myc* mice might be due to selection for cells with retroviral integration near the *Mcl1* locus. Using ligation-mediated PCR, we cloned 67 retroviral integration sites from *Myc* AML mice (n = 3, 34 clones; Supplemental Table 1), and from control *MSCV-GFP*–transplanted mice (n = 3, 33 clones; Supplemental Table 2). We identified a total of 44 unique retroviral integration

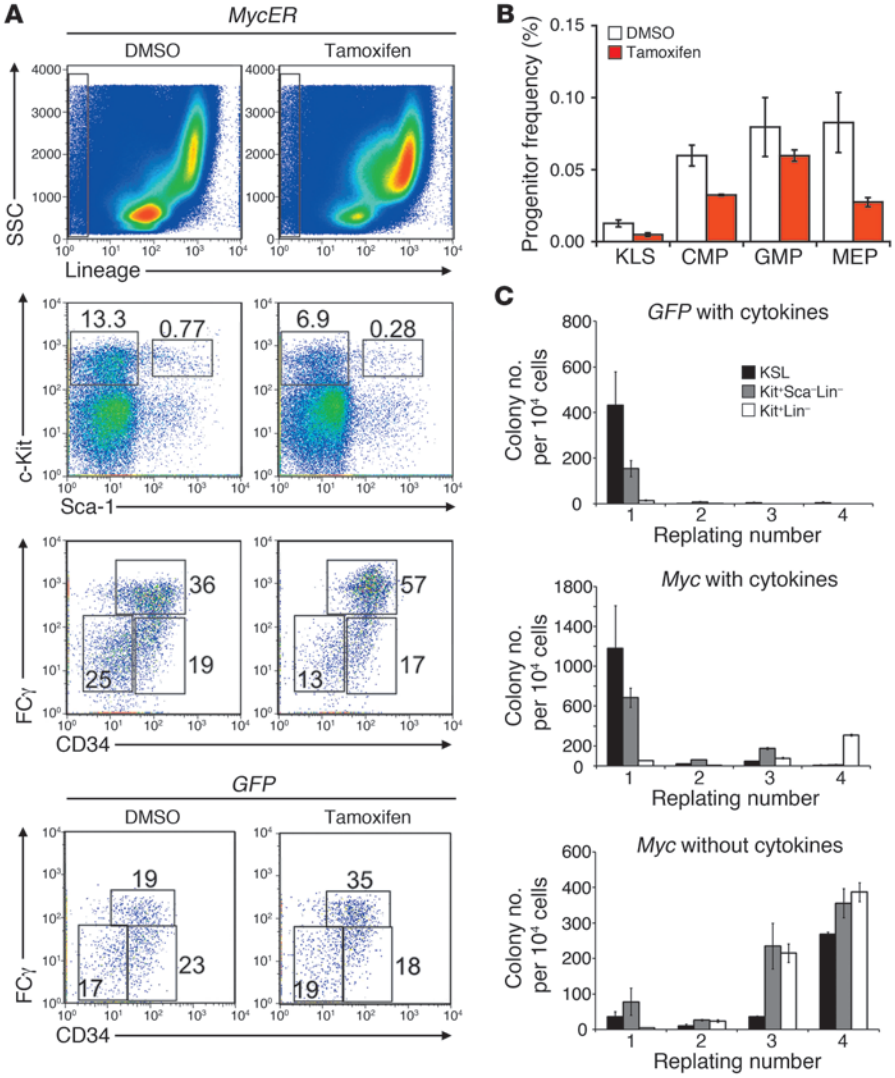

Figure 4
HSC and progenitor populations were not expanded in *Myc*-induced leukemic mice. (**A**) HSCs (KSL) were decreased, as was the total progenitor pool (Kit$^+$Sca$^-$Lin$^-$), after *MycER* induction for 7 days. CD34$^+$FCγ^+ CMPs were comparable between tamoxifen and DMSO treatment groups, a relative increase of CD34$^+$FCγ^+ GMPs was seen in *MycER* mice treated with tamoxifen, but also in control mice, and CD34$^-$FCγ^- MEPs were reduced after *Myc* induction. Representative flow cytometry plots are shown from mice transplanted with *MSCV-MycER* (n = 3) or *MSCV-GFP* (n = 3). Numbers within dot plots represent the percentage of events within the respective gates. (**B**) Frequencies of HSCs and progenitors in bone marrow of *MycER* mice. Absolute stem and progenitor cell numbers also decreased upon *Myc* induction (not shown). (**C**) Sorted KSL cells, a progenitor cell pool (Kit$^+$Sca$^-$Lin$^-$), and lineage-committed cells (Kit$^+$Lin$^+$) were transduced with *Myc* or *GFP* vector alone and plated in methylcellulose media in the absence or presence of cytokines. *Myc* induction increased cytokine-dependent progenitor colonies that failed to self renew. *Myc* also induced replatable cytokine-independent colony formation, and, surprisingly, Kit$^+$Lin$^+$ cell–derived colonies increased dramatically after third plating. Vector-transduced cells gave rise only to rare, non–self-renewing colonies (not shown). Kit$^-$Lin$^+$ cells were sorted but were substantially dead (Trypan blue–positive) after a single day and were not plated. Data in **B** and **C** are mean ± SD.

sites, none of which were close to the *Mcl1* locus. Notably, 86% (38 of 44) of the integration sites were nonrecurring. A single site of integration (*Prkcbp1* locus) was responsible for 71% (21 of 29) of the recurrent clones and was found in both *MSCV-Myc* (10 clones) and *MSCV-GFP* (11 clones) mice. These data are consistent with earlier Southern blot integration analysis (13) and support our model that the development of AML in the *MSCV-Myc* model is independent of retroviral integration site.

HSC and early progenitor cell populations are not expanded in Myc-induced AML. In WT mice, *Mcl1* is highly expressed in HSCs, and its expression level decreases in further differentiated progenitor cells: common myeloid progenitors (CMPs), common lymphoid progenitors (CLPs), megakaryocyte erythroid progenitors (MEPs), and granulocyte monocyte progenitors (GMPs) (19). One explanation for the elevated Mcl1 levels in *Myc* AML cells may be the relative increase of HSC and myeloid progenitor populations in *Myc*-induced AML. To monitor HSCs and progenitor cells in vivo, we retrovirally expressed inducible *MycER* into the bone marrow of WT mice using a bone marrow transplant assay (absent antiapoptotic mutations) and monitored the effects of *Myc* induction on myeloid lineages by flow cytometry. At 3 weeks after bone marrow transplant, robust donor engraftment was confirmed by flow cytometry (40%–60% GFP$^+$ peripheral mononuclear cells; data not shown). Recipient mice were then treated with tamoxifen or the carrier DMSO daily for 7 days to induce *Myc* expression, and bone marrow mononuclear cells were harvested for analysis. Forward versus side scatter profile of bone marrow with back gating to indicate cell lineages confirmed the expansion of myeloid cells in contrast to reduction of the B cell population (Figure 3E). Bone marrow stem and progenitor cell populations were then analyzed by flow cytometry. *Myc* induction did not significantly increase the relative or absolute numbers of Kit$^+$Sca$^+$Lin$^-$ (KSL) or immunophenotypically defined myeloid progenitor cells (e.g., CMPs, GMPs, and MEPs; Figure 4, A and B). There was a modest apparent increase in the relative abundance of GMPs (CD34$^+$FCγ^+) upon *Myc* induction, but this effect was nonspecific, as it was observed in *MSCV-GFP* control mice treated with tamoxifen as well (Figure 4A). Thus, *Myc* induction did not significantly increase HSC and myeloid progenitor pools in vivo, and Mcl1 elevation was not caused by increased HSCs and myeloid progenitors after *Myc* activation.

Myc confers self-renewal ability to committed myeloid progenitor cells. As *Myc* induction did not increase the percentage and absolute

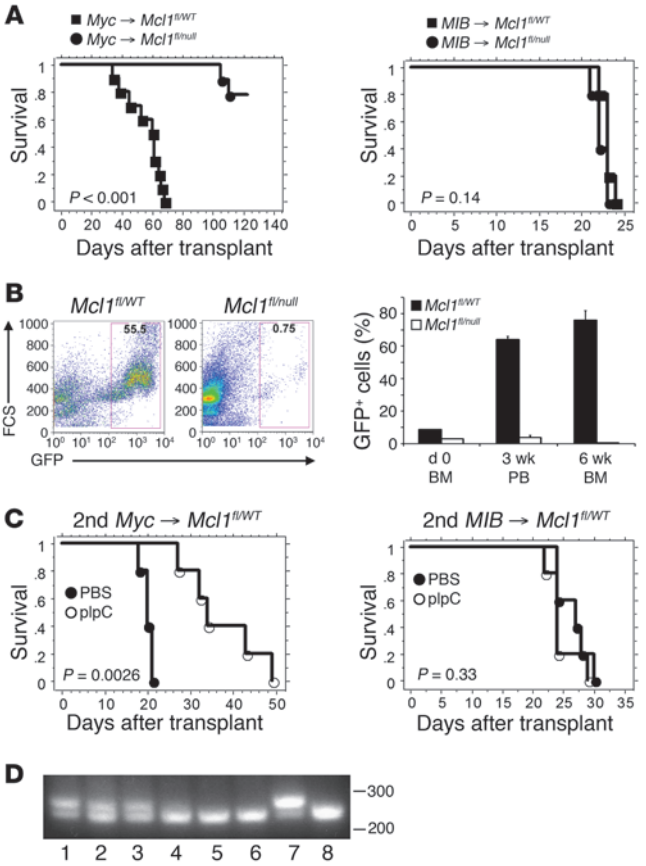

Figure 5

Mcl1 haploinsufficiency protects mice from *Myc*-induced AML. (**A**) Kaplan-Meier survival analyses of mice transplanted with *Myc* or *MIB*. Congenic *Mcl1*$^{fl/WT}$ or *Mcl1*$^{fl/null}$ mice were used as bone marrow donors as indicated. Note the differences in time scale between the graphs. (**B**) *Mcl1*$^{fl/null}$ cells failed to support transformation by *Myc*. GFP$^+$ cells in *Mcl1*$^{fl/WT}$ versus *Mcl1*$^{fl/null}$ recipients were determined by FACS before transplantation in bone marrow, at 3 weeks after transplantation in peripheral blood (PB), and at 6 weeks after transplantation in bone marrow. FCS, forward scatter. Numbers denote the percentage of events falling within the respective gate. Data are mean ± SD. (**C**) *Mx1-Cre*–mediated deletion of 1 *Mcl1* allele from established AML cells significantly prolonged the survival of secondary recipients. Leukemia cells from primary recipients of *Mx1-CreMcl1*$^{fl/WT}$ bone marrow transduced with *Myc* or *MIB* were transplanted into secondary recipients treated with pIpC or PBS. (**D**) PCR amplification of the 390-bp *Mcl1*^{fl} and 340-bp *Mcl1*^{WT} alleles from transplanted mice. Lane 1, *Myc Mcl1*$^{fl/WT}$ primary transplant; lane 2, *MIB Mcl1*$^{fl/WT}$ primary transplant; lane 3, *Myc Mcl1*$^{fl/WT}$ secondary transplant after pIpC treatment; lane 4, *MIB Mcl1*$^{fl/WT}$ secondary transplant after pIpC treatment; lane 5, *MIB Mcl1*$^{fl/WT}$ secondary transplant after pIpC treatment (spleen cells); lane 6, B6 129 *Mcl1*$^{fl/WT}$ pIpC-treated spleen cells; lane 7, B6 129 *Mcl1*$^{fl/WT}$ untreated control; lane 8, C57B6 WT control.

number of early hematopoietic cells (Figure 4A), we assessed its effect on self renewal using methylcellulose serial replating assays. Normal KSL cells, early progenitors (Kit$^+$Sca$^-$Lin$^-$), and lineage-committed cells (Kit$^+$Lin$^+$) isolated from WT mice were transduced with *Myc* or vector alone and then plated in methylcellulose either with or without cytokines. *GFP*-transduced KSL, early progenitor, and lineage-committed cells failed to form colonies after second plating in methylcellulose culture in the presence or absence of cytokines (Figure 4C and data not shown). In the presence of cytokines, *Myc* expression dramatically increased the number of initial colonies in KSL, early progenitors, and lineage-committed cells and conferred the ability to form colonies after multiple serial platings (Figure 4C). Notably, *Myc* expression conferred cytokine-independent colony formation activity to stem/progenitor cells and even to committed myeloid cells. These colonies could be serially replated in cytokine-free methylcellulose medium up to 5 times (Figure 4C and data not shown), which suggests that *Myc* is capable of conferring self-renewal activity to myeloid progenitor cells, including lineage-committed cells.

Mcl1 haploinsufficiency abrogates Myc-induced AML in mice. To genetically test whether elevated *Mcl1* expression is required for *Myc*-induced AML development, we expressed *Myc* into bone marrow cells derived from *Mcl1* gene–targeted normal mice and monitored disease development. As anticipated, *Myc* induced rapid fatal AML (mean survival, 56 ± 5 days) in *Mcl1* WT (*Mcl1*$^{fl/WT}$) mice (which were phenotypically identical to the phenotype previous reported; ref. 13) that was characterized by leukocytosis, splenomegaly, and hind limb paralysis with 100% penetrance (Figure 5A). However, *Myc* failed to cause disease in *Mcl1*-haploinsufficient (*Mcl1*$^{fl/null}$) recipients (Figure 5A). We monitored the transduced (GFP$^+$) cells in peripheral blood and bone marrow at 3 and 6 weeks after transplantation, respectively. In *Mcl1*$^{fl/null}$ recipients, GFP$^+$ cells were only 3.9% ± 1.3% in peripheral blood (versus 64% ± 2.1% in *Mcl1*$^{fl/WT}$ recipients) and less than 3% in bone marrow (versus greater than 90% in *Mcl1*$^{fl/WT}$ recipients; Figure 5B). As reported previously (19), complete loss of *Mcl1* expression (i.e., *Mcl1*$^{null/null}$) caused significant loss of early hematopoietic progenitor populations, including HSC and bone marrow ablation, whereas *Mcl1*$^{fl/null}$ mice had relatively normal hematopoiesis and HSC/progenitor populations. To control for the possibility of a previously unrecognized bone marrow phenotype in *Mcl1*$^{fl/null}$ mice, we repeated our bone marrow transduction-transplantation experiments coexpressing *Bcl2* with *Myc* (i.e., *MIB* to *Mcl1*$^{fl/WT}$ and *MIB* to *Mcl1*$^{fl/null}$), and all recipients developed leukemia with rapid disease latency (Figure 5A and data not shown).

To further elucidate the role of *Mcl1* in *Myc*-induced leukemia, we measured the effect of deleting 1 copy of *Mcl1* in fully transformed leukemia cells. We harvested leukemia cells from moribund *Mx1-Cre*–expressing *Mcl1*$^{fl/WT}$ mice transduced by *Myc* alone or *MIB* and injected them into secondary recipients treated with polyinosinic-polycytidylic acid (pIpC) to mediate Cre deletion or with control PBS. Deletion of a single *Mcl1* allele from *Myc*-induced *Mcl1*$^{fl/WT}$ leukemia cells significantly prolonged the survival in secondary recipient mice treated with pIpC compared with that in the PBS-treated mice ($n = 5$, $P = 0.0026$; Figure 5C). In contrast, deletion of 1 copy of *Mcl1* from *MIB*-induced *Mcl1*$^{fl/WT}$ leukemia cells had no

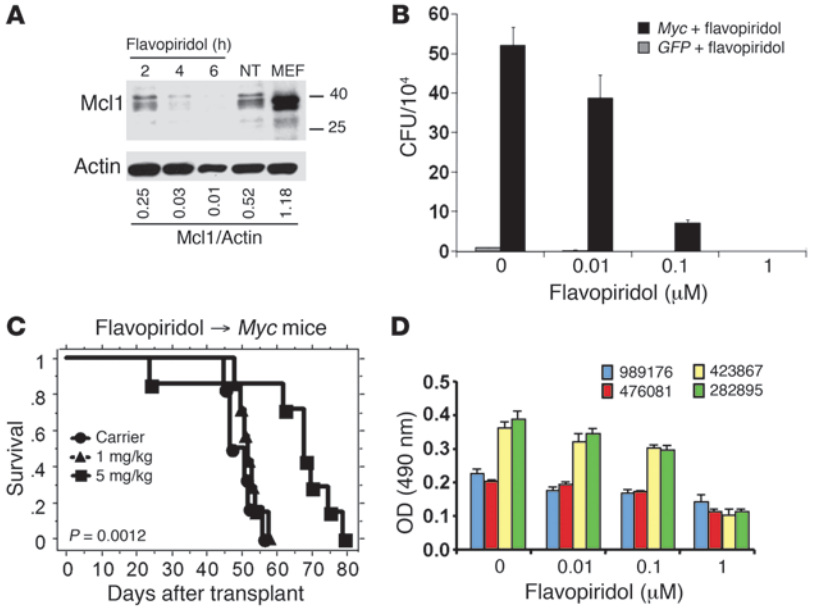

Figure 6
Pharmacologic suppression of Mcl1 expression inhibits growth of murine and human AML cells. (**A**) Western blot of Mcl1 protein levels in lysates prepared from *MSCV-Myc* AML cells treated in liquid culture for the indicated times with flavopiridol. NT, nontreated cells at 6 hours. Mouse embryonic fibroblasts were used as protein control. Ratio of Mcl1 to actin protein levels, quantified by densitometry, are shown below. (**B**) Dose-dependent flavopiridol suppression of *MSCV-Myc* cytokine-independent methylcellulose colony growth. At flavopiridol doses less than 1 μM, growth of normal cytokine-dependent bone marrow progenitors was not affected (not shown). (**C**) Survival of mice secondarily transplanted with *MSCV-Myc* AML cells was significantly prolonged by flavopiridol treatment. (**D**) MTT assay measurement of primary human AML cell growth in the presence of flavopiridol. Results from 4 separate patient samples, plated in triplicate, are shown. Data in **B** and **D** are mean ± SD.

effect on the survival of the secondary recipients treated with pIpC compared with PBS-treated mice (n = 5, P = 0.33; Figure 5C). To determine the mechanism by which *Myc*-induced leukemia progressed in $Mcl1^{fl/WT}$ mice, we examined the success of recombination in leukemic mice. Leukemic cells from *Myc* mice revealed an unrecombined $Mcl1^{fl}$ allele, whereas *MIB* cells demonstrated complete excision (Figure 5D). These data suggest that *Myc*-induced leukemic cells in pIpC-treated secondary mice were selected from a small population of cells harboring unrecombined WT *Mcl1*. These results demonstrate that the antiapoptotic function of *Mcl1* plays a crucial role in *Myc*-induced leukemia development and maintenance in mice.

Finally, we sought to assess the potential of MCL1 as a target for drug therapy in AML patients. Flavopiridol, a semisynthetic flavonoid, is a protein kinase inhibitor that effectively reduces *Mcl1* transcription in a variety of cell contexts (20). We found that flavopiridol rapidly and significantly decreased Mcl1 protein levels in bone marrow AML cells from leukemic *MSCV-Myc* mice (Figure 6A) and suppressed the growth of *MSCV-Myc* cells ex vivo (Figure 6B). Flavopiridol also significantly prolonged the survival of mice secondarily transplanted with *MSCV-Myc* AML cells (Figure 6C). Drug treatment also suppressed the growth of cultured primary human AML cells ex vivo in a dose-dependent manner (Figure 6D). These data provide support for the development of MCL1-targeted therapies in patients with AML.

Discussion

MCL1 is implicated in the pathogenesis of B cell malignancies, including B cell lymphoma (21, 22), multiple myeloma (23), and chronic lymphocytic leukemia (24), but the role of MCL1 in myeloid malignancy remains poorly defined. MCL1 is a unique BCL2 family member, characterized by rapid induction and degradation in response to extracellular stimuli that encompass a large range of growth factors, cytokines, and inducers of apoptosis (15). MCL1 expression is carefully regulated by RNA transcription, posttranscriptional splicing, posttranslational cleavage, and phosphorylation (25). Kaufmann et al. found that MCL1 protein levels are heterogeneous in AML patient cells, but that the levels of MCL1 protein expression increase in samples at the time of disease relapse after chemotherapy (26). Any antiapoptotic BCL2 family member can cooperate with *Myc* in leukemogenesis (27), but to our knowledge, the specific functional significance of MCL1 expression in AML has not been demonstrated previously.

We found high-level *MCL1* transcript expression in 111 of 111 untreated de novo AML patient bone marrow samples, including all AML subtypes, and observed abundant MCL1 protein expression in 6 of 7 AML patient samples examined (Figure 1). The antiapoptotic family members *BCL2* and *BCL-XL* were also expressed in patient AML samples, albeit less consistently. Expression of BCL2 has been associated with poor prognosis in AML (3). The BCL2 family members are likely to be functionally redundant in cancer (27). Our data suggest that upregulation of *MCL1* is an early event in AML pathogenesis, and *BCL2* and *BCL-XL* upregulation may occur later during the process of clonal evolution. Our gene profiling data suggested that *MCL1* might play an important role in AML pathogenesis, and we next evaluated the role of *Mcl1* in a murine model of AML.

We examined the expression of apoptosis-regulating proteins and identified a unique antiapoptotic profile in leukemic blast cells isolated from moribund AML mice (Figure 2A). Among the differences observed, Mcl1 protein was uniformly expressed at higher levels in AML cells than in normal or lymphoid leukemia cell populations (Figure 2B). We focused on the role of Mcl1 in our murine model because of the high *MCL1* transcript expression levels observed in human AML cells (Figure 1). Recent work has suggested that in a subset of AML patients, activating mutations in the FLT3 receptor tyrosine kinase may induce high MCL1 expression (28, 29). What mechanisms explain the high-level Mcl1 protein expression in *Myc*-induced AML cells? We favor the hypothesis that *Myc* overexpression upregulates *Mcl1* expression in myeloid progenitor cells indirectly. *Myc* induction directly suppresses the expression of Bcl2 and Bcl-xL antiapoptosis family members in both primary myeloid and lymphoid cells (30, 31). We found that activation of *Myc* in different cell types did not markedly increase

Mcl1 transcript or protein levels at early time points (Figure 3, B and C), which demonstrated that *Mcl1* is not a direct transcriptional target of *Myc*. Instead, we consistently observed increased *Mcl1* expression only after prolonged expression of *Myc* (Figure 3D), indicative of an indirect mechanism of *Mcl1* induction.

The composition of the bone marrow was dramatically altered by *Myc* induction (Figure 3E). We found significant differences in the way myeloid and lymphoid bone marrow lineages respond to *Myc* activation, which may explain why high-level *Myc* expression induces exclusively myeloid-lineage leukemia in this AML model (13). After 7 days of *Myc* induction, absolute numbers of B-lineage cells declined, whereas the absolute number of myeloid lineage cells rose substantially (Figure 3E). Murphy et al. recently found that lower dosages of *Myc* can promote cell growth without inducing apoptosis (32), but *MSCV-Myc*–reconstituted mice expressed *Myc* at higher levels in myeloid leukemia cells relative to other cell types (e.g., B lymphoid cells), which suggests that the rapid induction of AML by retroviral expression of Myc proteins is unlikely to be explained by subthreshold Myc protein levels.

To determine whether *Myc* expression was selecting for a Mcl1-expressing bone marrow population, we next examined the effects of *Myc* induction on HSC and progenitor cell populations (Figure 4). HSCs expressed *Mcl1* at higher levels compared with defined myeloid progenitor cell populations (e.g., CMPs and GMPs; ref. 19), and we thought that *Myc* induction might expand the HSC population. Instead, *Myc* induction caused HSC and progenitor cell populations to decline (Figure 4, A and B). The shift we observed, from HSCs and progenitor cells to more mature (i.e., Lin^+) cells, was consistent with the role of *Myc* as a promoter of cell differentiation (33), but failed to explain the development of leukemia in this model. In a parallel experiment, we transduced purified HSC and progenitor cell populations with *Myc* and assayed transformation by methylcellulose colony assays. Remarkably, *Myc* conferred self-renewal activity to more mature lineage-committed progenitor cells (Figure 4C). Leukemia-associated oncogenes have the ability to transform progenitor cells (34). Taken together, these data suggest that high Mcl1 expression in more mature committed myeloid progenitor cells cooperates with oncogene expression to induce AML.

Using *Mcl1* gene-targeted mice as donors for bone marrow transduction-transplantation experiments, we demonstrated that haploinsufficiency of *Mcl1* was sufficient to significantly protect mice from the development of *Myc*-induced AML. *Mcl1* heterozygous bone marrow cells transduced with *Myc* failed to maintain high *Myc* expression levels, and mice transplanted with $Mcl1^{fl/null}$ cells were extremely resistant to AML development (Figure 4). In a second set of experiments, AML was generated using $Mcl1^{fl/WT}$ donor cells, and leukemic bone marrow from these moribund mice was transplanted into secondary recipients treated with pIpC. The excision of a single *Mcl1* allele from *MSCV-Myc* AML cells significantly prolonged the survival of secondary recipient mice. Coexpression of *Bcl2* restored the ability of *Myc* to induce fatal leukemias in $Mcl1^{fl/null}$ cells, which demonstrated that *Mcl1* loss did not otherwise affect the target cells of leukemic transformation. Although the bulk of our data point to absolute dependence on Mcl1 for protection from oncogene-induced apoptosis, this remains to be formally proven, particularly with regard to primary human AML cells.

Our finding that AML development and maintenance were significantly dependent on *Mcl1* gene dosage has important clinical implications. *Mcl1* is required for normal bone marrow hematopoiesis, and conditional, complete knockout of *Mcl1* causes hematopoietic failure in mice (19). No phenotype has previously been reported in *Mcl1* heterozygous mice (19), but we found here that in the setting of *Myc*-induced AML, the survival of mice was significantly prolonged by reducing Mcl1 dose by 50%. Complete inhibition of Mcl1 would likely have profound effects on normal hematopoiesis, and our data suggest that a therapeutic window exists in which partial reduction of *Mcl1* dosage may inhibit leukemogenesis without affecting normal hematopoiesis.

These data support the development of novel MCL1-targeted agents for AML patients. The *MSCV-Myc* murine AML model may be useful for characterizing candidate pharmaceutical compounds for *Mcl1* activity. Small-molecule inhibitors targeting the BH3-binding groove of prosurvival BCL2 family members (i.e., BH3 mimetics) are currently in clinical development (5, 35). Preclinical studies have suggested that BH3 mimetics may have activity in AML and that responses to these compounds may depend on the expression patterns of individual BCL2 family members (36). Additional studies are required to identify AML patient subsets in which specific BCL2 family members are relevant, and may require the development of methods to quantify expression of different BCL2 family members at the protein level in patient samples. Moreover, small-molecule inhibitors may have activity in AML by targeting *MCL1* expression indirectly. For example, inhibiting the PI3K/AKT signal transduction pathway using the mammalian target of rapamycin (mTOR) inhibitor rapamycin represses *Mcl1* transcription (37), and flavopiridol, an inhibitor of cyclin-dependent kinases (CDKs; ref. 38), rapidly downregulates *Mcl1* levels (20, 39). We found that flavopiridol modestly suppressed the growth of unscreened primary human AML cells (Figure 6D). The response of human AML cells to any treatment is susceptible to individual patient genetics, and future work will allow us to identify patients that will benefit specifically from Mcl1-targeted drugs.

Methods

Plasmid DNA constructs and retrovirus production. Retroviral constructs *MSCV-Myc-Ires-GFP* and *MIB* (i.e., *MSCV-Myc* plus *Bcl2*) have been described previously (13). *MycER* was provided by G. Evan (UCSF, San Francisco, California, USA) and was subcloned into *MSCV-IRES-GFP* retroviral vector backbone.

Replication-incompetent retroviral supernatant was made according to the calcium coprecipitation protocol as described previously (13). Retroviral titers of *MSCV-Myc* and *MSCV-MycER* was determined by flow cytometry analysis of the GFP^+ cells in retrovirally transfected 3T3 cells, and MIB viral titer was determined by measuring the copy number of proviral *Psi*-sequence in transfected cells by real-time PCR.

Bone marrow transduction and transplantation. Bone marrow cell harvesting, transduction, and transplantation were carried out according to the method previously reported (13). BALB/c and C57BL/6 donor mice (6–7 weeks old) were obtained from Jackson Laboratories. *Mcl1* conditional knockout mice ($Mx\text{-}1CreMcl1^{fl/WT}$ and $Mx1\text{-}CreMcl1^{fl/null}$; referred to herein as $Mcl1^{fl/WT}$ and $Mcl1^{fl/null}$, respectively) were provided by J. Opferman (St. Jude Children's Research Hospital, Memphis, Tennessee, USA). Mononuclear bone marrow cells were spinfected twice, on days –1 and 0 (13). Bone marrow cells (1×10^6) were injected i.v. into lethally irradiated syngeneic BALB/c (800 cGy), C57BL/6, or C57BL/6 129 (1,200 cGy) mice. Secondary transplants were performed by injecting i.v. 5×10^5 unsorted mononuclear spleen cells isolated from moribund primary transplant recipients into secondary syngeneic recipient mice treated with 500 cGy gamma

irradiation. To induce recombinase activity in the secondary recipients of *Mcl1*$^{fl/WT}$ *Myc* leukemia cells, 400 μg pIpC (Sigma-Aldrich) was injected i.p. into each recipient mouse daily for 3 days before irradiation.

Induction of Myc in vivo. Unfractionated murine bone marrow cells were transduced with *MSCV-MycER* retrovirus and subsequently transplanted into syngeneic recipient mice. Engraftment was documented at 3 weeks after transplant using flow cytometry detection for robust GFP expression in peripheral blood cells. Mice were then treated once daily with tamoxifen (0.5 mg/mouse) for 7 days by i.p. injection. In vitro induction of *Myc* was performed by applying 4-OH tamoxifen to culture medium to a final concentration of 250 nM.

Mouse analysis. Recipient mouse monitor, tissue, and mononuclear cell preparation and staining were carried out as described as previously (13). Kaplan-Meier and significance analyses were performed using Statview software (SAS Institute). Fluorescence-conjugated antibodies were used to recognize lineage markers of Gr-1, Mac-1, B220, IgM, CD4, and CD8 (BD Biosciences — Pharmingen).

For analysis of progenitor populations, the unfractionated bone marrow cells were stained as described previously (40). Briefly, PerCP-Cy5.5–conjugated CD3e (clone 145-2C11), CD4 (clone RM4-5), CD8a (clone 53-6.7), CD19 (clone 1D3), CD45R (clone RA3-6B2), TER119 (clone TER-119), CD127 (clone SB/199), and GR1 (clone RB6-8C5) were used for lineage markers (BD Biosciences — Pharmingen). Cells were also stained using biotin-conjugated CD34 (clone RAM34), PE-conjugated streptavidin, allophycocyanin-conjugated Sca-1 (clone D7), PE-Cy7–conjugated CD16/32 (clone 93), and allophycocyanin–Alexa Fluor 750–conjugated CD117 (clone 2B8) antibodies obtained from eBiosciences. Data was collected from 5×10^6 cells using a MoFlo flow cytometer (Dako) and was analyzed using FloJo software (Tree Star). Studies in animals were approved by the Washington University Animal Study Committee (St. Louis, Missouri, USA).

Methylcellulose colony assays. Whole bone marrow cells from 6- to 8-week-old C57BL/6 mice (Taconic Farms) were prepared and transduced as described above. HSCs and progenitor cells were sorted from whole bone marrow cells, stained as described above, and subsequently plated into 12-well cell culture plates (approximately 1–63 × 10^4 cells/well) and transduced with retroviral supernatants by incubation for 12 hours at 32°C in transplant media as described above. Cells were then transduced again by centrifugation at 900 *g* for 90 minutes in the presence of 5 μg/ml polybrene (American Bioanalytical) and allowed to recover at 37°C for 3 hours.

The colony formation assay was carried out using 12-well untreated cell culture plates and either cytokine-containing (SCF, IL-3, IL-6, or EPO) methylcellulose medium MethoCult M3434 (StemCell Technology) or cytokine-free medium MethoCult M3234 (StemCell Technology). For unfractionated bone marrow cells, 4×10^4 cells/well for MethoCult M3434 and 4×10^5 cells/well for MethoCult M3234 were plated in triplicate. For sorted hematopoietic cells, cells were washed twice with PBS and plated into MethoCult M3434 (400 KSL, 800 $Kit^+Sca^-Lin^-$, and 2×10^4 Kit^+Lin^+ per well) or MethoCult M3234 (4,000 KSL, 8,000 $Kit^+Sca^-Lin^-$, and 2×10^5 Kit^+Lin^+ per well). Colonies containing at least 30 cells were counted 9 days after plating. The GFP^+ colony was counted under fluorescent reverse microscope. For serial replating analysis, pooled colonies were washed 3 times with PBS and replated into same methylcellulose medium (MethoCult M3434, 4×10^4 cells/well; MethoCult M3234, 4×10^5 cells/well) in triplicate. Colonies were counted 9 days after replating.

Flavopiridol treatment. Flavopiridol (Sigma-Aldrich) was prepared in water, and the flavopiridol concentrations were determined by serial dilution. The final concentrations of 0, 0.01, 0.1, and 1 μM were used for liquid cultures of *Myc* mouse AML cells and primary human AML cells and for colony formation culture of *Myc* AML cells. MTT assay used to evaluate the effect of flavopiridol on human AML cells has been described preciously (41). The primary human AML cells used in this assay were provided by T. Fehniger (Washington University School of Medicine, St. Louis, Missouri, USA). Flavopiridol was diluted with PBS and injected i.p. into *Myc* secondary recipient mice.

Western blot analysis. Protein lysate preparation and Western blots were performed following standard protocols as described previously (13). Antibodies used to stain the membrane were as follows: Myc (catalog no. sc-788), Mcl1 (catalog no. sc-819), Bcl2 (catalog no. sc-7382), Noxa (catalog no. sc-22764), and PUMA (catalog no. sc-20534) from Santa Cruz Biotechnology Inc.; Bcl-xL (catalog no. 610211) from BD Biosciences — Pharmingen; XIAP (catalog no. 2042) from Cell Signaling Technology; Bim (catalog no. 202000) from Calbiochem; and Bak (catalog no. 06-536) from Upstate. To assess protein loading, blots were stripped and reprobed with anti–β-actin antibody (Sigma-Aldrich).

Expression profiling and data analysis. AML patient samples were obtained from patients with de novo AML, following informed consent, as described previously (42). Human subject research was approved by the Washington University Human Research Project Office (HRPO) and the Siteman Cancer Center Protocol Monitoring and Review Committee (PRMC). Bone marrow RNA samples were generated from 111 AML patients that fulfilled criteria including age greater than 18 years, at least 30% bone marrow involvement by leukemia, at least 2 cytogenetic abnormalities, and no previous therapy.

Bone marrow aspirates were obtained from consented de novo AML patients, and RNA was prepared from unfractionated snap-frozen cell pellets. Total cellular RNA was purified using TRIzol reagent (Invitrogen), quantified using UV spectrometry (Nanodrop Technologies), and qualitatively assessed using a RNA NanoChip assay with BioAnalyzer 2100 (Agilent Technologies). Samples were labeled and hybridized to Affymetrix Human Genome U133 Plus 2.0 Arrays (Affymetrix) using standard protocols in the Siteman Cancer Center Multiplexed Gene Analysis Core Facility. The experiments were performed in triplicate. To perform interarray comparisons, the raw scan data from each array were scaled to a target intensity of 1,500 using statistical algorithm MAS 5 in Affymetrix GCOS version 1.2 software. Scaled data for each array were merged with updated gene annotation data for the array. Data visualization and further analysis were performed with functional genome in the Decision Site (Spotfire).

According to the most updated annotations available from EntrezGene, UniGene, and Gene Ontology databases and manual curation, we identified 174 apoptosis- and AML-related probe sets on the U133 Plus 2.0 array. Average signal intensity of these probe sets was calculated for each patient group, and the top 25 upregulated probe sets were plotted.

RT-PCR. RNA was extracted from cells using TRIzol (Invitrogen). cDNA transcription was performed using the first strand synthesis kit (Invitrogen) per the manufacturer's instructions. RNA (1 μg) was used in each 20-μl reaction system. For semiquantitative PCR or real-time PCR, 1 μl cDNA production from each reaction and 150 pM 5′ primer and 150 pM 3′ primer was used in a 25-μl reaction system. Primers were as follows: *ODC* forward, 5′-TTGCCACTGATGATTCCAAA-3′; *ODC* reverse, 5′-GAACTTCAGGGTCAGCTTGC-3′; *GFP* forward, 5'GGACGTGGTTT TCCTTTGAA-3′; *GFP* reverse, 5′-AGCCACCACCAATATCAAGC-3′; *Mcl1* forward, 5′-GGTGCCTTTGTGGCCAAACACTTA-3′; *Mcl1* reverse, 5′-ACCCATCCCAGCCTCTTTGTTTGA-3′; *Actin* forward, 5′-GCTGTATTCCCCTCCATCGTG-3′; *Actin* reverse, 5′-CACGGTTGGCCTTAGGG TTCAG-3′. In semiquantitative PCR, reaction was terminated every 2 cycles beginning at the 16th cycle, which was determined by preliminary experiments. *GFP* was used as loading control. Real-time PCR was carried out using a SYBR Green real-time PCR system (Applied Biosystem). β-Actin was used as cDNA quantity calibration. Primers for

Mcl1^{fl} PCR were 5′-CTGAGAGTTGTACCGGACAA-3′ and 5′-GCAGTACAGGTTCAAGCCGATG-3′.

Ligation-mediated PCR and integration site analysis. Retroviral integration sites were analyzed in DNA samples isolated from splenocytes derived from either *MSCV-Myc* leukemic mice or *MSCV-GFP* control mice using ligation-mediated PCR (Supplemental Methods).

Statistics. Statistical comparisons were made using Student's 2-sided *t* test. *P* values of 0.05 or less were considered significant.

Acknowledgments

This work was supported by the American Cancer Society (grant RSG-06-169-01-LIB to M.H. Tomasson and H. Luo) and the NIH (grant PO1 CA101937 to M.H. Tomasson and Z. Xiang). We are grateful to Julie Ritchey for technical assistance in viral integration site analysis and to Daniel Link, Timothy Graubert, John F. DiPersio, Katherine Weilbaecher, and Mathew Walter for critical discussion and review of the manuscript.

Received for publication May 26, 2009, and accepted in revised form February 3, 2010.

Address correspondence to: Michael H. Tomasson, Washington University School of Medicine, Campus Box 8007, 660 South Euclid Avenue, St. Louis, Missouri 63110, USA. Phone: 314.362.9350; Fax: 314.362.9333; E-mail: tomasson@wustl.edu.

Zhifu Xiang's present address is: Division of Hematology and Oncology, Department of Internal Medicine, Winthrop P. Rockefeller Cancer Institute, University of Arkansas for Medical Science, Little Rock, Arkansas, USA.

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