Mcl1 haploinsufficiency protects mice from Myc-induced acute myeloid leukemia

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Antia apoptotic 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
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 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.
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
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
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–Fcy– HSCs and DMSO treatment groups, a relative increase of CD34–Fcy– GMPs was seen in MycER mice treated with tamoxifen, but also in control mice, and CD34–Fcy– 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, 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.

Figure 4

HSC and progenitor cell 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–Fcy– HSCs and DMSO treatment groups, a relative increase of CD34–Fcy– GMPs was seen in MycER mice treated with tamoxifen, but also in control mice, and CD34–Fcy– 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, 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.

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 anti-apoptotic 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 4E). 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–Fcy–) 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

sites, none of which were close to the Mcll locus. Notably, 86% (38 of 44) of the integration sites were nonrecurring. A single site of integration (Prkcep1 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.
A 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+ Sca1− 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 (Mcl1fl/WT) mice (which were phenotypically identical to the phenotype previously 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 (Mcl1fl/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 Mcl1fl/null recipients, GFP+ cells were only 3.9% ± 1.3% in peripheral blood (versus 64% ± 2.1% in Mcl1fl/WT recipients) and less than 3% in bone marrow (versus greater than 90% in Mcl1fl/WT recipients; Figure 5B). As reported previously (19), complete loss of Mcl1 expression (i.e., Mcl1fl/null/fl) caused significant loss of early hematopoietic progenitor populations, including HSC and bone marrow ablation, whereas Mcl1fl/null mice had relatively normal hematopoiesis and HSC/progenitor populations. To control for the possibility of a previously unrecognized bone marrow phenotype in Mcl1fl/null mice, we repeated our bone marrow transplantation experiments coexpressing Bcl2 with Myc (i.e., MIB to Mcl1fl/WT and MIB to Mcl1fl/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 Mcl1fl/WT mice transduced by Myc alone or MIB and injected them into secondary recipients treated with polynucleoside-polycytidylic acid (pIpC) to mediate Cre deletion or with control PBS. Deletion of a single Mcl1 allele from Myc-induced Mcl1fl/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 Mcl1fl/WT leukemia cells had no
A small population of cells harboring unrecombined WT phosphorylation (25). Kaufmann et al. found that MCL1 protein expression is carefully regulated by RNA 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, McI1 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 transplantation/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 Mcl1fl/fl cells were extremely resistant to AML development (Figure 4). In a second set of experiments, AML was generated using Mcl1fl/fl 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 Mcl1fl/fl 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 were determined by flow cytometry analysis of the GFP+ cells in retrovirally transduced 3T3 cells, and MIB viral titer was determined by measuring the copy number of proviral Pu-sequence in transduced cells by real-time PCR.

Bone marrow transplantation and transplantation. Bone marrow cell harvesting, transplantation, 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 (Mx1-CreMcl1fl/fl and Mx1-CreMcl1fl/fl, referred to herein as Mcl1fl/fl and Mcl1fl/fl, 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 × 10^6 unsorted mononuclear spleen cells isolated from moribund primary transplant recipients into secondary syngeneic recipient mice treated with 500 cGy gamma-rays.
irradiation. To induce recombinate activity in the secondary recipients of McI/WT Myc leukemia cells, 400 μg plpC (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 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 eBioscience. 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 1–6 × 10^5 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 Kt/Sca Lin, and 2 × 10^6 Kit/Lin′ per well) or MethoCult M3234 (4,000 KSL, 8,000 Kit/Sca Lin, and 2 × 10^6 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 0.01, 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 previously (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), McI (catalog no. sc-819), Bel2 (catalog no. sc-7382), Noxa (catalog no. sc-22764), and PUMA (catalog no. sc-20534) from Santa Cruz Biotechnology Inc.; Bel-xl (catalog no. 61021) from BD Biosciences—Pharmingen; XIAP (catalog no. 2042) from Cell Signaling Technology; Bim (catalog no. 20200) 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 S′ primer and 150 pM 3′ primer was used in a 25-μl reaction system. Primers were as follows: ODC forward, 5′-TTGCCACTGTAGATTCCCAA-3′; ODC reverse, 5′-GAACTTCAAGGTCAGTTGC-3′; GFP forward, 5′-GGGACGGTGTGTTCCTTTGGA-3′; GFP reverse, 5′-AGCCACACCAAATCATCAAGC-3′; McI forward, 5′-GGTGCCCTTGTGGCCCAACATTA-3′; McI reverse, 5′-ACCCTACGACGCTTTGTTGTA-3′; Actin forward, 5′-GCTGTATTCCCCCTCCACTGGT-3′; Actin reverse, 5′-CAGGGTTGCGGTCTTTAGGTT-3′. In total, 25 reverse transcriptions were performed with real-time 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 MeTH-
Mcl1\(^{\text{p}}\) PCR were 5'-CTGAGAGTTGATCCGGGACA-3' and 5'-GCAGTA-CAGGGTTCAAGCCCATG-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.

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