c-Myc rapidly induces acute myeloid leukemia in mice without evidence of lymphoma-associated anti-apoptotic mutations

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Abstract

Ectopic expression of c-Myc (Myc) in most primary cell types results in programmed cell death, and malignant transformation cannot occur without additional mutations that block apoptosis. The development of Myc-induced lymphoid tumors has been well studied and supports this model. Myc can be upregulated in acute myeloid leukemia (AML), but its exact role in myeloid leukemogenesis is unclear. To study its role in AML, we utilized a Murine Stem Cell Virus (MSCV) retroviral gene transfer/transplantation system to broadly express Myc in the bone marrow of mice either alone or in combination with anti-apoptotic mutations. Myc expression in the context either of Arf/Ink4a loss or Bcl-2 co-expression induced a mixture of acute myeloid and acute lymphoid leukemias (AML+ALL). In the absence of anti-apoptotic mutations however, all MSCV-Myc transplanted mice (100%, N=110) developed AML exclusively. MSCV-Myc-induced AML was polyclonal, readily transplantable, possessed an intact Arf-p53 pathway, and did not display cytogenetic abnormalities by spectral karyotyping (SKY) analysis. Lastly, we found that Myc preferentially stimulated the growth of myeloid progenitor cells in methylcellulose. These data provide the first direct evidence that Myc is a critical downstream effector of myeloid leukemogenesis and suggest that myeloid progenitors are intrinsically resistant to Myc-induced apoptosis.
Introduction

Deregulation of the proto-oncogene c-Myc (MYC) is considered one of a series of oncogenic events required for mammalian tumorigenesis\(^1\). \(\text{MYC}\) encodes a basic helix-loop-helix leucine zipper transcription factor, that dimerizes with its partner, Max, and regulates multiple cellular functions including cell cycle, cell growth, differentiation, apoptosis, metabolism and angiogenesis via transcription of downstream target genes\(^2\). The Myc/Max dimer can also repress transcription of another set of target genes through a less well-understood mechanism\(^5,6\). The c-Myc proto-oncogene is involved in transformation and cell proliferation in part via activation of the \(\text{cyclin D2}\) promoter\(^7\), but also induces programmed cell death, mediated by NRF-1\(^8\) and the Arf-p53 pathway\(^9,10\). Forced expression of Myc in primary cells is generally thought to induce growth arrest or apoptosis\(^11,12\).

The oncogenic function of c-Myc has been best studied in the \(\text{E}_\mu\)-\(\text{Myc}\) transgenic mouse, in which c-Myc expression is targeted to the lymphoid compartment by the immunoglobulin heavy chain gene promoter and enhancer\(^13\). In \(\text{E}_\mu\)-\(\text{Myc}\) mice, expression of \(\text{Myc}\) is not sufficient to cause leukemia. A latency period of 4-6 months is required for the accumulation of cooperating mutations before lymphoma can develop\(^14\). The majority of the \(\text{E}_\mu\)-\(\text{Myc}\) tumors harbor mutations in the now canonical Arf-Mdm2-p53 pathway\(^14,15\). Although the p53 pathway controls many functions including cell-cycle, DNA damage response and apoptosis, Bcl-2 or dominant-negative caspase 9 completely also cooperate with Myc, and alleviate the pressure to inactivate p53 during lymphomagenesis\(^15\). Therefore, of the many functions of the Arf-Mdm2-p53 pathway, it is the ability to mediate apoptosis that is targeted by cooperating mutations in \(\text{Myc}\)-induced lymphomagenesis.

\(\text{MYC}\) dysregulation, via a variety of mechanisms, has also been associated with myeloid leukemias\(^16\). Double minute chromosomes in patients with acute myeloid leukemia (AML) contain \(\text{MYC}\) amplifications\(^17,18\). C-Myc expression is apparently required for the oncogenic effects of the Philadelphia chromosome product, BCL-ABL\(^19\), and over-expression of c-Myc also complements the transfor-
mation defects of BCR-ABL mutants\textsuperscript{20}. A recent study showed that many important oncogenes in myeloid leukemogenesis including AML1-ETO, PML-RAR\textalpha and PLZF-RAR\textalpha induce leukemogenesis by activating c-Myc\textsuperscript{21}, suggesting c-Myc is a downstream target of these oncogenes. Lastly, c-Myc is upregulated by activating mutations in the gene encoding the FLT3 receptor tyrosine kinase, found in nearly one third of all patients with AML\textsuperscript{22,23}.

There is a lack of useful animal models for studying the role of Myc in myeloid leukemia. AML infrequently develops in \textit{E\mu SR-Myc} mice, in which a heterologous promoter inducibly drives Myc expression in bone marrow cells, however most of these animals develop T-cell lymphomas\textsuperscript{24}, making it a cumbersome system to study myeloid disease. Retroviral transduction of c-Myc into p53-null bone marrow cells rapidly causes lymphoid leukemias, but not myeloid disease\textsuperscript{25}. Finally, v-Myc transduction can induce myeloid leukemias in a retroviral transduction/transplantation system\textsuperscript{26,27}, however v-Myc bears point mutations known to contribute synergistically to transforming potential measured in culture\textsuperscript{28}, so results obtained using v-Myc cannot be extrapolated to un-mutated c-Myc.

Using the murine stem cell virus (MSCV) system to broadly express Myc in primary murine bone marrow cells, we compared the effects of ectopic Myc expression upon myeloid and lymphoid progenitors in methylcellulose colony assays, and found a preferential increase in myeloid colonies. We used two different retroviral bone marrow transduction/ transplantation systems to examine the contribution of anti-apoptotic mutations to the development of Myc-induced disease in mice. In the setting of either \textit{Ink4a} loss or Bcl-2 coexpression, Myc induced both myeloid and lymphoid leukemias. In contrast, rapidly fatal, disseminated myeloid leukemia developed independently of anti-apoptotic mutations. We analyzed AML cells from \textit{MSCV-Myc} mice extensively and found no evidence of spontaneous anti-apoptotic mutations. Our results provide direct evidence of Myc’s role in myeloid leukemogenesis and suggest that myeloid progenitor cells are relatively resistant to Myc-induced apoptosis.
Methods

Plasmid constructs

To make the MSCV constructs that express c-Myc, the cDNAs encoding murine c-Myc (Myc), (provided by Michael Cole, Princeton University) were subcloned into the Xho site in the MSCV-ires-GFP vector (provided by Warren Pear, University of Pennsylvania). The MSCV-Bcl2 construct was made by subcloning the XhoI/EcoRI fragment of Bcl2 (provided by Carlo Croce, Thomas Jefferson University, Philadelphia) into Xho-EcoRI sites of MSCV-ires-GFP. To make the MSCV construct that express both Myc and Bcl-2, Bcl-2 was first amplified using primers with NcoI and Cla sites engineered. The ampli-con was purified and cut with those enzymes and put into NcoI-ClaI digested MSCV-GFP (replacing the GFP). c-Myc was then added into XhoI site as above.

Ecotropic retroviral gene transfer and murine bone marrow transplants

Replication incompetent supernatant was made by transiently transfecting 293T cells with each construct and ecotropic packaging sequence pIK6.1MCV.ecopac.UTD (Ecopac, M. Finer Cell Genosys, Redwood City, CA). Supernatants were harvested 48 hours post transfection, passed through a 0.45 micron filter and kept in frozen aliquots at -80°C. Retroviral titers were determined by measuring percentage of GFP positive 3T3 cells or quantitation of proviral DNA in infected cells using real-time PCR specific for MSCV Psi-sequences (protocol available on request). Bone marrow cells transduction and transplantation were carried out according to the method previous reported. Briefly, nuclear bone marrow cells were harvested from 6-7 week old BALB/c, B6 129 or B6 129-Cdkn2atm1Rdp donor mice (Jackson Labs, Bar Harbor, ME) treated with 0.45mg 5-flourouracil (5-FU, Sigma, St. Louis, MO). Spinfection was performed twice on D -1 and D 0. 1x10^6 cells were injected i.v into lethally irradiated (on Day-1) syngeneic Balb/c (800 cGy) or B6 129 (1200 cGy) mice. Secondary transplants were per-
formed by intravenously injecting unsorted nuclear spleen cells isolated from a moribund primary transplants into secondary recipient mice treated with 500 cGy gamma irradiation syngeneic recipient.

**Analysis of mice**

Mice were monitored 3 times per week for disease by palpation and observation. Peripheral blood was obtained by either retro-orbital phlebotomy after adequate methoxyflurane (Springvale, Australia) anesthesia or femoral phlebotomy using heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA). Nuclear cell suspensions of spleen, lymph notes or thymus were made by passing tissue through nylon mesh cell strainers (Falcon, Lincoln Park, NJ) wetted with PBS. Tissues from the mice were stored in a 10% buffered formalin solution (Sigma, St. Louis MO). Kaplan Meier and significance analysis was performed using Statview software (SAS Institute, Inc, Cary, NC).

**Detection of provirus and clonality assays**

Genomic DNA was isolated from mouse spleen and bone marrow using the Puregene DNA Isolation Kit (Gentra, Minneapolis, MN) per manufacture's instructions. 10μg of DNA was digested with XbaI (cuts once in each LTR and releases a 4.2kb provirus band). To assess clonality, genomic DNA was digested with Bgl II (cuts once in provirus). DNA was precipitated and subjected to agarose gel electrophoresis and transferred to a nylon membrane (Hybond-N Plus, Amersham Pharmacia, Piscataway, NJ). After a 2 hour pre-hybridization, the blot was hybridized for 24 hours at 65°C with an 800bp GFP (HindIII- NcoI) probe labeled with dα32P ATP using the Random Primed DNA labeling kit (Roche Diagnostics, Indianapolis, IN) per manufacturer's instructions. Membranes were washed with 2X SSC 2X 5 minutes, 2X SSC, .1% SDS 3 X 10 minutes at 65°C and exposed to radiographic film overnight or a week at -80°C. Blots were stripped by incubation in boiling 0.1% SDS, and incubating until SDS reached room tem-
perature. The \textit{Ink4a} exon1\(\beta\) probe was kindly provided by Ron DePinho and Ned Sharpless, Harvard Medical School.

\textit{Immunophenotyping}

One million freshly made nuclear cells were suspended in 100ul Flow buffer and incubated 30 min. on ice with appropriate antibodies including: phycoerythrin (PE) conjugated Gr-1, PE-B220, PE-CD4, Biotin-Mac-1, Biotin-CD3, Biotin-CD8, Biotin-CD43 and Biotin-IgM (all from Becton Dickenson, San Diego, CA). Samples were then incubated with 1ul of Streptavidin in 100ul Flow Buffer for Biotin samples for 30 min before analyzed on a Cytomation Modulation Flow Cytometry machine.

\textit{Sequence analysis.}

RNA was isolated from BALB/c and MSCV-Myc mice using the RNeasy kit (Qiagen, Valencia, CA) per manufacturer's instructions. cDNA was made using a Superscript First Strand Synthesis System (In-vitrogen, Carlsbad, CA) per manufacturer's instructions. PCR reactions for P53 and Arf were performed using, 2ul cDNA, 2mM final MgCl\(_2\), 10uM final primer concentration, 10X PCR Reaction buffer (Fisher Scientific, Pittsburgh, PA), 1unit Taq DNA Polymerase (Fisher Scientific, Pittsburgh, PA). Thermal Program: 94°C 2 min., 39 cycles of: 94°C 30 seconds, 71.5°C 1 min, 72°C 1 min. Ink4a amplification was the same except for 1mM final MgCl\(_2\) and annealing temp of 66°C. P53 primers: TRP53F3 GCGGGGTTGCTGGGATTG; TRP53R2 CCG CGGATCTTGAGGGTGAAATAC TRP53F4: TGGCCCCTGTCATCTTTTGTCC; \textit{Arf} primers: P19F1: GAGGCCGCCGCTGAGGGAGTA; P19R1: CTAAGAAGAAAAAGCGGGCTGAG; \textit{Ink4a} primers: INK4A 2F: CCCCACGGCCCGAACTCT; INK4A 2R: AAGGCGGGCTGAGGCAGGATT. Products were gel purified using the QIAEX II Gel Extraction kit (Qiagen, Valencia, CA) per manufacturer's instructions. Sequences were compared to normal BALB/c P53. (GI 200198), p19\textit{Arf} (accession number nm_009877), and p16\textit{Ink4a} (GI 3002946). 8 mice for \textit{Arf} were sequenced. Data ranges from base pair 2
to 586. (1= A from the ATG start codon). 6 mice were sequenced for Ink4a. Data ranges from base pair 85 to 525. For P53, sequence data was collected from 8 mice. Data spans base pair -113 to 347. Two mice were sequenced at the C terminal end, and data ranges from bp 654-952.

Western blots.

Protein lysates were made from sorted GFP positive cells or unsorted bone marrow, spleen and lymph node cells from affected and control animals with lysis buffer containing protease inhibitors (Cell Signaling, Beverly, MA) and 1mM PMSF (Sigma, St. Louis). 20ug Protein samples were resolved on a 9% SDS polyacrylamide resolving gel and transferred to a nitrocellulose membrane (Midwest Scientific, St. Louis, MO). After incubated in 5% blocking solution (5% Non-fat dried milk, 150mM NaCl, 50mM Tris pH8) at room temperature for one hour, Membranes were incubated with primary antibody 4°C overnight, washed 3 times 15 minutes with TBST (10mM Tris, pH7.6, 150mM NaCl, 0.05% Tween 20) and then incubated with secondary antibody for 40 min. room temperature. Blots were subsequently washed 3X 20 minutes with TBST. Antibodies: P53 (1:250) and P19 antibodies (1:500) were kindly provided by Jason Weber, Washington University St. Louis MO. P21 (1:250), Bcl-2 (1:1000), c-Myc (1:1000)(Santa Cruz Biotechnology, Santa Cruz, CA). Bcl-xl (1:500) (Cell Signaling Technologies). β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) or actin (Sigma, St. Louis MO)(1:5000). Secondary Antibodies (1:5000) HRP conjugated anti-rabbit (Upstate Biotechnology, Lake Placid, NY) or anti-mouse (Sigma, St. Louis). Blots were developed using the Pierce Enhanced Chemiluminescence system (Pierce, Rockford, IL) and exposed to Kodak Biomax film (SciMart, St. Louis, MO).

Methylcellulose culture

B6 129 mice were transplanted with either MSCV GFP or MSCV MycER GFP transfected bone marrow cells following the method described above. Eight to ten weeks later, GFP-positive bone marrow cells from the recipient mice were plated in methylcellulose media containing myeloid cytokines (SCF, IL-3,
IL-6, Epo), lymphoid cytokines (IL-7), or no cytokines (Stemcell Technologies). 4x10^4 cells/well for myeloid culture, 4x10^5 for lymphoid and non-cytokines culture were plated in triplicate in each experiment. 200nM 4-hydroxy-tamoxifen (Sigma) or ethanol carrier was added when the culture was set up. 7 day later the colony numbers were counted and the cells in each culture were subjected to immunophenotype analysis.
Results

Myc rapidly induces acute myeloid leukemia (AML), independent of Ink4a status

To characterize the effects of c-Myc on hematopoietic progenitor cells in vivo, we performed bone marrow transduction/transplantation experiments using an MSCV retrovirus encoding the wild-type c-Myc (MSCV-Myc). Unfractionated bone marrow was harvested from wild type mice or mice bearing a targeted deletion in Ink4a exon 2A that results in the loss of expression of both p19Arf and p16Ink4a proteins (Ink4a^-/-). MSCV-Myc mice transplanted using Ink4a^-/- bone marrow (MSCV-Myc Æ Ink4a^-/-) developed a rapidly fatal disease characterized by significant peripheral leukocytosis with circulating myeloblasts and lymphoblasts, splenomegaly, lymphadenopathy, hind-limb paralysis (Figure 1A and Table 1). To our surprise, MSCV-Myc mice transplanted with Ink4a^+/+ bone marrow (MSCV-Myc Æ Ink4a^+/+) also succumbed to disease rapidly (median survival = 61 days) with splenomegaly and hind-limb paralysis.

Blood leukocytosis and lymphadenopathy was conspicuously absent/reduced (Figure 1A and Table 1). Notably, non-saturating retroviral insertion with replication-incompetent MSCV-GFP virus did not cause disease either in wild-type or tumor prone Ink4a^-/- mice during a 200-day observation (Figure 1A).

We used flow cytometry to compare the immunophenotypes of GFP-positive cells from MSCV-Myc Æ Ink4a^+/+ and MSCV-Myc Æ Ink4a^-/- mice. MSCV-Myc Æ Ink4a^-/- bone marrow was packed with two distinct populations of malignant cells: one with features of myeloblasts and another with features of lymphoblasts. The immunophenotypic studies confirmed the lineage of these two populations: one stained double positive with Gr-1 and Mac-1 representing myeloid origin and another stained positive with B220 representing B-cell lymphoid origin (Figure 1B). These two malignant populations composed over 90% of bone marrow. All of the MSCV-Myc Æ Ink4a^-/- transplants developed significant lymphadenopathy and the GFP positive cells from their lymph nodes were exclusively immature CD43^+IgM^- B lymphocytes (Figure 1C, D). All of the MSCV-Myc Æ Ink4a^-/- transplants also developed thymus enlargement. Pathology revealed infiltration of the thymus with lymphoblasts and immunophe-
notyping showed these were immature B-lineage lymphoblasts (Figure 1E). In summary, mice transplanted with Ink4a−/− bone marrow expressing Myc (MSCV-Myc → Ink4a−/−) developed a myeloid and lymphoid leukemias simultaneously.

In contrast, the 100% of the MSCV-Myc → Ink4a+/+ transplants developed acute myeloid leukemia exclusively (Figure 1 and Table 1). The MSCV-Myc → Ink4a+/+ transplants developed splenomegaly and hind-limb paralysis, but did not develop significant lymphadenopathy or peripheral leukocytosis to the same degree as Ink4a−/− mice (Table 1). Immunophenotypic studies of the GFP positive bone marrow cells showed a dominant cell population that stained double positive with Gr-1 and Mac-1, confirming their myeloid lineage, and a conspicuous reduction in the size of the B220+ lymphoid population (Figure 1B). Analysis of the lymph nodes revealed two distinct populations: a high GFP population stained positive with Gr-1 and Mac-1, representing myeloid leukemic cells infiltrating lymph nodes; and group of GFP-low cells that expressed either B220 or CD3, and were indistinguishable from normal, mature lymphocyte populations (Figure 1C, D, E). Microscopic analysis of the tissues of MSCV-Myc → Ink4a+/+ mice revealed bone marrow packed with myeloblasts with abundant cytoplasm, fine chromatin and prominent nucleoli (Figure 1F) and these leukemic cells infiltrated spleen, liver, and nervous system leading to hind limb paralysis (Supplementary data). Histologic examination of lymph nodes from MSCV-Myc mice, revealed infiltration of large malignant cells with characteristic myeloblast morphology, normal appearing lymphocytes, and an absence of transformed lymphoblasts (Figure 1F). Therefore, targeted disruption of the Ink4a locus cooperated with Myc in our model system to achieve malignant transformation of lymphoid cells. In the setting of wild-type Ink4a, MSCV-Myc mice lived 33 days longer (Table 1) and displayed no evidence of lymphoid malignancy. However, Myc-expressing wild-type Ink4a mice did succumb uniformly to acute myeloid leukemia (AML).
Coexpression of Bcl-2 is required for Myc-induced lymphoid but not myeloid leukemia

To further study the role of apoptosis in MSCV-Myc AML, we made additional retroviral constructs designed to co-express Myc with the anti-apoptotic protein Bcl-2 (Figure 2A). We confirmed that these constructs expressed Myc and Bcl-2 proteins (Figure 2B). Consistent with published reports, coexpression of Myc and Bcl-2 together, but not either gene alone, transformed Ba/F3 cells to factor-independence (Figure 2C).

To assess the effect of apoptosis suppression on Myc-induced leukemogenesis, we performed bone marrow transduction/transplantation assays with Myc alone, Bcl-2 alone, or both together. Successful retroviral transduction was confirmed in all experiments by flow cytometry of GFP positive cells in peripheral blood three weeks after transplantation (with the exception of mice transplanted with the GFP-less MSCV-Myc+Bcl2 construct (Table 1). Notably, retroviral transduction of the Bcl2 protooncogene by itself was insufficient to induce disease in a single animal, consistent with published data. The analyses of bone marrow, lymph nodes, spleen and thymus from MSCV-Bcl2 mice showed normal morphology microscopically and normal immunophenotype even several months post-transplantation (Figure 3F). In sharp contrast, all MSCV-Myc+Bcl-2 mice (n=33, 100%) developed a rapidly fatal leukemia/lymphoma characterized by leukocytosis, hind limb paralysis, splenomegaly and lymphadenopathy and a median survival of 16 days (Figure 3A and Table 1). Flow cytometric analysis demonstrated that, in the bone marrow of MSCV-Myc+Bcl2 mice, Mac1/Gr-1 double positive cells predominated (Figure 3B), but thymus and lymph node tissues were also heavily infiltrated with large B220+ lymphoblastic cells (Figure 3C, D, E). Further analysis of these B220 positive cells showed that these represent immature B lymphocytes that stained negative for surface IgM (Figure 3E). Consistent with these data, examination of affected tissues from MSCV-Myc+Bcl2 mice demonstrated two morphologically distinct leukemic populations: the bone marrow of MSCV-Myc+Bcl-2 mice was packed with myeloblasts, while the lymph nodes of these mice were packed with lymphoblasts (Figure 3F).
Myc and Bcl-2 expression cooperate to transform primary lymphoid cells\textsuperscript{30,32}, however, even in the absence of transgenic Bcl-2 expression, \textit{MSCV-Myc} mice universally (n=35, 100\%) developed ruffled fur, cachexia, splenomegaly and hind limb paralysis. Median survival of \textit{MSCV-Myc} mice was 47 days following marrow transplantation (Figure 3A, Table 1). Immunophenotype studies showed that the majority of bone marrow cells from \textit{MSCV-Myc} mice stained positive for the myeloid markers Mac-1 and Gr-1 (Figure 3B). Although the composition of the bone marrow of \textit{MSCV-Myc+Bcl2} and \textit{MSCV-Myc} was similar (Figure 3B), analysis of the lymph nodes and thymus of leukemic animals revealed stark contrasts. The lymphoid organs of \textit{MSCV-Myc} contained only myeloid blasts and immunophenotypically mature lymphoid cells. The immature B lymphoblasts dominant in \textit{MSCV-Myc+Bcl2} mice were not detected in \textit{MSCV-Myc} mice (Figure 3C, D, E).

We did observe a small highly autofluorescent population in the bone marrow of \textit{MSCV-Myc} mice (weakly B220 and CD3 double positive) that stained strongly with Annexin V and 7-ADD, with cytoplasmic blebbing and nuclear fragmentation upon histologic examination (not shown). Despite this evidence of apoptosis, the bone marrow cavity of all \textit{MSCV-Myc} mice analyzed was also packed with neoplastic myeloid blasts, with expansion of cells to the surrounding soft tissue and musculature (Figure 3F). Hind limb paralysis was due to invasion by malignant cells from vertebral body marrow cavities into the spinal canal (Supplementary data). Malignant cells were also found disrupting the architecture of the spleen, and in perivascular foci in the liver (Supplementary data). Differential counts of May-Grunwald-stained bone marrow cytospins demonstrated elevated myeloid blast counts (greater than 30\%) with promyelocytes or more mature neutrophils (at least 10\% of nucleated bone marrow cells) characteristic of AML of the M2 subtype (AML with maturation, Figure 3F, and data not shown).

Moribund \textit{MSCV-Myc} mice, in contrast to \textit{MSCV-Myc+Bcl2} mice, had no significant lymphadenopathy or leukocytosis (Table 1). Histological examination of lymph node cells revealed small ma-
ture-appearing lymphocytes with normal morphology (Figure 3F) consistent with the immunophenotypic analysis (Figure 3D, E). The thymuses from MSCV-Myc mice were of normal size and were found to contain normal lymphocytes predominantly (Figure 3D, E). However, histological studies revealed a small population of cells with myeloid leukemic morphology (Figure 3F).

We performed secondary transplantation experiments to assess the self-renewal capacity of leukemic cells from our mice. We isolated mononuclear cells from the spleens of moribund primary MSCV-Myc or MSCV-Myc+Bcl2 animals and intravenously injected serial dilutions into sub-lethally irradiated syngeneic mice. Both MSCV-Myc+Bcl2 mixed lineage and MSCV-Myc myeloid leukemias were easily transplantable into secondary recipients. Secondary recipients succumbed to leukemias with phenotypes identical to the primary animals (data not shown), and the median survival of these mice was inversely correlated with the number of cells injected, allowing an estimation of the frequency of leukemia initiating cells. The number of leukemia initiating cells was greater than 1 in 500 in MSCV-Myc+Bcl2 mice and between 1 in 5000 to 1 in 500 in MSCV-Myc mice (Figure 4). These findings demonstrate that leukemic cells from both MSCV-Myc+Bcl2 and MSCV-Myc mice have the capacity to self-renew, and suggest that differences in disease latency between MSCV-Myc mice and MSCV-Myc+Bcl2 mice (16 vs. 47 days) may be due in part to the presence of a greater number of transformed clones in the setting of anti-apoptotic mutations, (e.g. lymphoid plus myeloid versus myeloid alone).

We also used secondary transplantation to functionally assess the normal-appearing cells detected in the lymphoid organs of MSCV-Myc mice. Unfractionated thymocytes or lymph node cells from moribund MSCV-Myc → Ink4a−/− or MSCV-Myc+Bcl2 mice gave rise to lymphoid leukemias in secondary recipients, whereas thymocytes or lymph node cells from MSCV-Myc mice either failed to cause disease in secondary recipients, or gave rise to myeloid lineage leukemias due to contaminating myeloblasts (Table 2). Therefore, in contrast to malignant lymphoid cells transformed by Myc in the setting
of anti-apoptotic mutations, lymphocytes from mice expressing Myc alone, while potentially “pre-malignant,” were not able to cause disease in secondary recipients.

We assessed apoptosis in our leukemic mice, and found robust Myc-induced apoptosis in the bone marrow of moribund MSCV-Myc mice with AML. Apoptosis was notably reduced in the marrow of leukemic MSCV-Myc+Bcl2 and MSCV-Myc→Ink4a−/− mice (Figure 5a). Similar results were obtained using TUNEL staining of diseased tissues (Figure 5b). Taken together, our data demonstrate that while transformation of lymphoid cells requires the active inhibition of apoptosis, Myc is able to transform immature myeloid cells, and induce fatal AML, without suppression of apoptosis by genetic manipulation.

**Myc-induced AML is polyclonal and maintains an intact Arf-p53 pathway**

Myc expression can elicit genomic instability, raising the possibility that Myc may induce tumorigenesis by facilitating the accumulation of mutagenic chromosomal abnormalities. To examine the MSCV-Myc myeloid leukemia genome for chromosomal abnormalities, we examined metaphase spreads from the spleen tumors of five animals by spectral karyotyping (SKY). No clonal chromosomal abnormalities were identified in any of the tumors examined (Figure 6A). Analysis of proviral integration was also performed to determine the clonality of MSCV-Myc tumors. We examined genomic DNA isolated from the splenocytes for the presence of proviral sequences by Southern hybridization using a provirus-specific probe. Using a restriction endonuclease that cuts twice in the provirus, genomic DNA isolated from spleen mononuclear cells was subjected to Southern blot analysis using an EGFP probe. A band of the expected size was found in all affected tissues from MSCV-Myc mice, but not in genomic DNA from control animals (Figure 6B, left panel). Using an enzyme that cuts only once in the provirus, analysis of the same genomic DNAs revealed that none of the tumors analyzed (n>20) were clonally derived. Instead, repeated analysis of multiple tumors demonstrated a heterogeneous pattern of faint bands at vary-
ing molecular weights, suggesting an polyclonal or oligoclonal tumor cell population (Figure 6B, right panel).

In $E\mu$-Myc lymphomas, loss of p19Arf expression occurs frequently due to biallelic deletion of the Ink4aArf locus. To characterize the Ink4aArf locus in our MSCV-Myc leukemias, we subjected gDNA from leukemic tissues to Southern analysis using a probe specific for Ink4a exon 1\(\beta\). We found the Ink4aArf locus to be in the germline configuration in all tumors tested (n=6 primary, Figure 7A; n=4 secondary, data not shown). Although we detected no large scale deletions in the Ink4a locus, smaller deletions or point mutations affecting Ink4a or Arf would also be expected to contribute to tumorigenesis. To address this possibility, we generated cDNA using bone marrow RNA from MSCV-Myc transplants and sequenced the coding regions of both Arf (n=8) and Ink4a (n=6) genes. In every case, PCR amplification generated amplicons of the expected size, and no mutations were found in transcripts encoding p16Ink4a or p19Arf (data not shown). We also sequenced p53 from cDNA amplified from MSCV-Myc tumors, and again, in every tumor tested (n=8), we found amplicons of the expected size. In 4/4 mice, we detected a silent C358T that likely represents an unreported polymorphism of our Balb/c substrain, but no base changes that would result in amino acid substitution or premature stop. Although we found no somatic mutations in p53, Arf or Ink4a in any of our MSCV-Myc tumors, the wild-type germline Balb/c Ink4a allele has been linked to plasmacytoma susceptibility and encodes a hypomorphic p16^{Ink4a} protein$^{34}$. We excluded this as an explanation for the phenotype we observed in Balb/c mice by repeating MSCV-Myc bone marrow transduction/transplantation using bone marrow from Black6 mice, whose Ink4a allele encodes a fully functional protein. Black6 mice succumbed to an AML phenotype identical to that seen with Balb/c mice with similar median survival and 100% penetrance (data not shown).
We next examined protein extracts from \textit{MSCV-Myc} and other tumors for expression of Myc, p19\textsuperscript{Arf}, p16\textsuperscript{Ink4a}, p53, p21, Bcl-2 and Bcl-XL proteins (Figure 7B-F). Mutations in modifier genes such as Dmp1, or methylation of Arf may functionally impair the Arf/Mdm/p53 pathway. To exclude the possibility that other genetic or epigenetic changes could be affecting p53 levels or function, Myc-expressing tumor cells from moribund \textit{MSCV-Myc} animals were purified using high-speed flow cytometry on the basis of GFP expression and forward and side scatter profiles to eliminate contamination from normal splenocytes. We subjected these purified MSCV-Myc tumor cells to 500cGy gamma-irradiation and analyzed levels of p53 and the p53 target gene p21\textsuperscript{waf/cip}. p53 levels were low, but uniformly detectable, in purified \textit{MSCV-Myc} tumor cells both before, and two hours after, gamma irradiation (Figure 7B). Notably, we did not see supra-physiologic expression of p53 in any of our samples as has been reported in association with inactivating mutations of p53\textsuperscript{14}. In all samples tested, p21\textsuperscript{waf/cip} levels were increased after irradiation compared to lysates harvested before irradiation (Figure 7B), demonstrating a functional p53 response. P19\textsuperscript{Arf} protein was also detected in all tumors tested, albeit at somewhat reduced levels in myeloid compared with lymphoid tumors (Figure 7D). Thus, despite detailed examination of the Arf-p53 pathway at the DNA, RNA and protein levels, no lesions in this tumor suppressor pathway were found in any of the tumors tested.

Examination of leukemic tissues by Western analysis also showed that the anti-apoptotic protein Bcl-2 was not expressed at detectable levels except in \textit{MSCV-Myc+Bcl2} tumors (Figure 7C and D). The Bcl-2 family member Bcl-xL was broadly expressed in \textit{MSCV-GFP} control bone marrow, as well as in unfractionated leukemia samples from \textit{MSCV-Myc} and \textit{MSCV-Myc+Bcl2} mice (Figure 7C). However, analysis of FACS purified \textit{MSCV-Myc} tumor cells revealed much lower levels of Bcl-XL protein (Figure 7E). Therefore, the Bcl-xL signal we observed in leukemic tissues is likely due to contaminating normal marrow elements. Finally, to determine if the AML phenotype we observed in \textit{MSCV-Myc}
mice was an artifact of extremely high expression from the MSCV LTR, we compared the level of c-Myc protein in our MSCV-Myc AML tumor lysates, with lysates made from an Eµ-Myc transgenic tumor. We found that our MSCV-Myc system does not express Myc more highly than the well-studied Eµ-Myc system. If anything, the Eµ-Myc tumor expressed Myc to a higher level than did our MSCV-Myc AML samples (Figure 7F).

c-Myc preferentially expands myeloid but not lymphoid progenitor cells ex vivo.

Lastly, we used methylcellulose colony assays to compare the effects of forced Myc expression upon progenitor cells of myeloid and lymphoid origin. We used FACS-purified, GFP-positive cells from mice that had been transplanted with bone marrow mononuclear cells expressing an inducible Myc allele (MSCV-MycER) or GFP alone (MSCV-GFP). Myc induction caused a significant increase in the number of myeloid progenitor colonies, and in the number of cytokine-independent progenitor colonies, but not in the number of lymphoid colonies (Table 3). To confirm the lineage of these colonies, we analyzed cells in aggregate by flow cytometry. Cells from colonies plated in myeloid cytokines, or no cytokines were predominantly Gr-1-positive, while cells plated in the lymphoid cytokine IL-7 were predominantly B220 positive (data not shown). These data support the model that myeloid progenitor cells are preferentially and cell autonomously expanded by Myc induction.
Discussion

We used a murine bone marrow transduction/transplantation system to broadly express Myc in the bone marrow of mice both in the presence and absence of anti-apoptotic mutations. When apoptosis was blocked, either by the absence of Ink4a, or by co-expression of Bcl-2, Myc induced a “bi-phenotypic” leukemia comprised of two distinct myeloid and lymphoid malignant cell populations (AML+ALL). In contrast, ectopic Myc expression in all mouse strains tested (Balb/c, Black6 and B6/129S) uniformly caused a rapidly fatal AML phenotype with 100% penetrance even in the absence of anti-apoptotic mutations. In humans with myeloid leukemias, MYC expression is dysregulated via multiple mechanisms including gene amplification, transcriptional regulation by fusion oncoproteins such as AML/ETO, and by activating mutations in receptor and non-receptor tyrosine kinases\textsuperscript{19,21,22}. Our results provide the first direct evidence of Myc’s central role as a downstream mediator of myeloid leukemogenesis.

Our data are consistent with the recent findings that the cellular effects of Myc are dependent upon the developmental context in which it is expressed\textsuperscript{35-37}. Two groups have also recently published data in regards to the role of Myc in modulating hematopoietic stem cell function, albeit with different results. One group demonstrates that Myc expression promotes self-renewal of stem cells\textsuperscript{38}, and the other proposes that Myc expression promotes differentiation at the expense of self-renewal\textsuperscript{39}. Our data support the idea that Myc promotes self-renewal, however additional experiments are required to exclude the possibility that Myc in our system is transforming a cell that already possesses self-renewal capability.

Hematopoietic stem and progenitor cells may avoid Myc-induced apoptosis by the expression of endogenous anti-apoptosis genes. The Bcl-2 family member Mcl-1 is expressed in developmentally immature hematopoietic cells and is critical for maintenance of stem and progenitor cells\textsuperscript{40}. The transcriptional repressor Slug is also expressed preferentially in immature hematopoietic cells and is re-
quired for protection of these cells from radiation-induced, p53-mediated apoptosis\textsuperscript{41}. Our model is that developmentally immature hematopoietic stem cells and/or myeloid progenitor cells are relatively apoptosis-resistant and therefore are especially susceptible to Myc-induced transformation. A more detailed understanding of the mechanisms by which immature bone marrow progenitor cells resist apoptosis and are transformed by Myc may allow us to identify novel, broadly applicable therapeutic targets for patients with AML.

**Acknowledgements**

We would like to thank Bill Eades, Tim Graubert and the Siteman Cancer Center Flow Cytometry Core for assistance with MoFlo cell sorting; Jan Nolta, Jesper Bonde, and David Hess for valuable reagents and help analyzing flow cytometry data; Steve Weintraub, Jason Weber, Troy Baudino, John Cleveland and Michael D. Cole for valuable reagents; Wanghai Zhang for help with TUNEL staining; Dan Link, Tim Graubert, Tim Ley and Katherine Weilbaecher for valuable discussion and critical reading of the manuscript.
References

33. Felsher DW, Bishop JM. Transient excess of MYC activity can elicit genomic instability and tumorigenesis. Proc Natl Acad Sci U S A. 1999;96:3940-3944


Table 1: Summary of primary bone marrow transplantation experiments

<table>
<thead>
<tr>
<th>Construct</th>
<th>Donor background</th>
<th>No.</th>
<th>Median survival (days)</th>
<th>GFP 3 week (%)</th>
<th>WBC (x10^3/ul)</th>
<th>Spleen weight (mg)</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myc</td>
<td>B6 129 Ink-/-</td>
<td>10</td>
<td>28±1.1</td>
<td>79.5±2.7</td>
<td>65.5±20.7</td>
<td>214±50</td>
<td>AML+ALL</td>
</tr>
<tr>
<td>Myc</td>
<td>B6 129 Ink+/+</td>
<td>10</td>
<td>61±3.7</td>
<td>56.2±3.9</td>
<td>6.0±1.4</td>
<td>380±40</td>
<td>AML</td>
</tr>
<tr>
<td>GFP</td>
<td>B6 129 Ink-/-</td>
<td>10</td>
<td>&gt;200</td>
<td>40.5±2.8</td>
<td>2.9±0.4</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>GFP</td>
<td>B6 129 Ink+/+</td>
<td>6</td>
<td>&gt;200</td>
<td>78.2±5.4</td>
<td>6.2±0.6</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Myc+Bcl2</td>
<td>BALB/c</td>
<td>33</td>
<td>16±0.7</td>
<td>N.A.</td>
<td>113.7±18.8</td>
<td>355±15</td>
<td>AML+ALL</td>
</tr>
<tr>
<td>Myc</td>
<td>BALB/c</td>
<td>35</td>
<td>47±2.9</td>
<td>86.9±0.6</td>
<td>12.3±1.5</td>
<td>386±30</td>
<td>AML</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>BALB/c</td>
<td>10</td>
<td>&gt;200</td>
<td>76.3±3.2</td>
<td>9.9±0.7</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

GFP indicates percentage of GFP-positive peripheral blood leukocytes measured by flow cytometry three weeks after marrow transplantation. N.A., not applicable. WBC, white blood count. AML, acute myeloid leukemia. ALL, acute lymphoid leukemia. MSCV-Myc+Bcl2 construct does not contain GFP.
### Table 2: Summary of results from secondary transplantation experiments using lymphoid tissues: lymphoid cells from MSCV-Myc mice are not fully transformed.

<table>
<thead>
<tr>
<th>Donor leukemic mice</th>
<th>Tissue</th>
<th>Mice No.</th>
<th>Mean survival (days)</th>
<th>Disease Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myc→Ink4a+/+</td>
<td>Thymocytes</td>
<td>3</td>
<td>34</td>
<td>Myeloid leukemia</td>
</tr>
<tr>
<td>MIB→BALB/c</td>
<td>Thymocytes</td>
<td>2</td>
<td>58</td>
<td>Mixed lineage leukemia</td>
</tr>
<tr>
<td>MIB→BALB/c</td>
<td>LN cells</td>
<td>3</td>
<td>37</td>
<td>Mixed lineage leukemia</td>
</tr>
<tr>
<td>Myc→BALB/c</td>
<td>LN cells</td>
<td>2</td>
<td>N.A.</td>
<td>None</td>
</tr>
</tbody>
</table>

Single cell suspensions containing 1-2x10^6 cells from indicated tissues of moribund primary animals were injected intravenously into sub-lethally irradiated syngeneic mice. Myc, MSCV-Myc; MIB, MSCV-Myc+Bcl2; LN, lymph node; N.A., not achieved.
Table 3: Bone marrow transduction with tamoxifen-inducible Myc stimulates myeloid colony growth in methylcellulose colony assays.

<table>
<thead>
<tr>
<th>Construct</th>
<th>4-OH-T</th>
<th>Myeloid</th>
<th>Lymphoid</th>
<th>No cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td>MycER</td>
<td>+</td>
<td>930±50</td>
<td>57±3</td>
<td>207±18</td>
</tr>
<tr>
<td>MycER</td>
<td>-</td>
<td>390±50</td>
<td>64±6</td>
<td>41±5</td>
</tr>
<tr>
<td>GFP</td>
<td>+</td>
<td>410±40</td>
<td>34±5</td>
<td>5±1</td>
</tr>
<tr>
<td>GFP</td>
<td>-</td>
<td>440±30</td>
<td>35±3</td>
<td>8±1</td>
</tr>
</tbody>
</table>

Bone marrow from mice transplanted with indicated retroviruses was sorted for GFP and plated in methylcellulose media containing myeloid cytokines (SCF, IL-3, IL-6, Epo; 5x10^4 cells/well), lymphoid cytokines (IL-7; 4x10^5 cells/well), or no cytokines (4x10^5 cells/well). Cells were plated in triplicate in each experiment. Myeloid colony numbers were multiplied by 10 to normalize for number of cells plated. Colony numbers are +/- standard deviation. 4-OH-T, 4-hydroxy-tamoxifen. A representative of four independent experiments is shown.
Figure Legends

Figure 1: MSCV-Myc induces myeloid and lymphoid disease phenotypes simultaneously in *Ink4a*<sup>-/-</sup> mice, and myeloid leukemia exclusively in *Ink4a*<sup>+/+</sup> mice.

(A) Kaplan-Meier survival analysis of mice transplanted with *Ink4a*<sup>+/+</sup> or *Ink4a*<sup>-/-</sup> bone marrow. GFP=MSCV-GFP, Myc=MSCV-Myc, Ink=Ink4a. Mice reconstituted with *Ink4a*<sup>-/-</sup> bone marrow transduced with GFP (*GFP→Ink*<sup>-/-</sup>*) were free of disease. Median survival of *Myc→Ink4a*<sup>-/-</sup> reconstituted mice was 28 days, and *Myc→Ink4a*<sup>+/+</sup> mice was 62 days. (B) Flow cytometric analysis of bone marrow cells from *Myc→Ink4a*<sup>+/+</sup> and *Myc→Ink4a*<sup>-/-</sup> mice. Bone marrow from *GFP→Ink4a*<sup>+/+</sup> mice are shown as controls. GFP-positive cells from *Ink4a*<sup>-/-</sup> mice show two populations of leukemic cells: a Mac-1<sup>+/</sup>Gr-1<sup>+</sup> myeloid population, and a B220<sup>+/</sup>/CD3- lymphoid population. In *Ink4a*<sup>+/+</sup> mice, there is only a single Mac-1<sup>+/</sup>/Gr-1<sup>-</sup> myeloid population. (C) Flow cytometric analysis of lymph node cells from *Myc→Ink4a*<sup>+/+</sup> and *Myc→Ink4a*<sup>-/-</sup> mice. Lymph node cells from *GFP→Ink4a*<sup>+/+</sup> mice are shown as controls. *Myc→Ink4a*<sup>-/-</sup> mice show a single GFP-positive population with a B-lymphoid immunophenotype (Mac-1/-/Gr-1-, and B220<sup>+/</sup>/CD3-). Lymph node cells from *Myc→Ink4a*<sup>+/+</sup> mice show two populations of cells, a GFP-low and a GFP-high population. The GFP-high cells are Mac-1<sup>+/</sup>/Gr-1<sup>+</sup> myeloid cells, and the GFP-low cells are a mixture of B220<sup>+</sup> B-lymphocytes, and CD3<sup>+</sup> T-lymphocytes similar to those seen in lymph nodes of control *GFP→Ink4a*<sup>+/+</sup> mice. (D) Further immunophenotyping of lymph node cells from leukemic mice. IgM and CD43 staining reveals that lymphoid cells from *Myc→Ink4a*<sup>-/-</sup> mice are immature (IgM-/CD43-low) B-lineage cells, in contrast to lymph node cells from *Myc→Ink4a*<sup>+/+</sup> mice and *GFP→Ink4a*<sup>+/+</sup> mice which are a mixture of mature IgM-postive B-cells, and T-cells that...
are CD3+. (E) Bar chart summarizing immunophenotype data from tissues of affected animals. (F) Cell morphology. *Myc → Ink4a−/−* mice show a mixture of myeloid and lymphoid blast cells in the bone marrow; and lymphoid blasts in the lymph nodes (LN) and thymus (Thy). *Myc → Ink4a+/+* mice show only myeloid blasts in the bone marrow and lymph nodes, and normal appearing cells in thymus.

**Figure 2: Design of retroviral constructs expressing Myc and Bcl-2.** (A) Murine c-Myc was inserted into the MSCV IRES GFP backbone, which uses the long terminal repeat (LTR) sequence to drive high expression. Two mutant forms of c-Myc were generated. One is a point mutant that changes amino acid 136 from a tryptophan to a glutamic acid (W136E). The other mutant is a complete deletion of the Myc Box II domain (amino acid 129-145). Dark bars represent Myc Box I and II. Striped bar represents the basic helix loop helix leucine zipper motif. Arrows represent LTRs. Psi represents the viral packaging sequence. B=BamHI, E=EcoRI, N=NcoI, H=HindIII, X=Xba. The murine Bcl-2 coding region was inserted into the viral backbone in the similar fashion. A construct co-expressing both Myc and Bcl-2 were generated by replacing GFP sequence in the Myc construct with Bcl-2 coding region. (B). Expression of Myc and Bcl-2 in transfected 3T3 cells. The cells in exponential growth phase express much higher endogenous c-Myc compared to those after serum starvation. Cells in exponential growth phase that were transfected with either GFP or Bcl-2 express similar levels of c-Myc. The cells transfected with either Myc or Myc/Bcl-2 construct express significantly higher levels of c-Myc. Only the cells transfected with either c-Myc or c-Myc/Bcl-2 express Bcl-2 protein. (C). IL-3 independent growth of BaF3 cells. Only cells transfected with both Myc and
Bcl-2 (black line), but not Myc or Bcl-2 alone (grey line and long dash line) grow in the absence of IL-3. Cells transfected with GFP alone do not grow in the absence of IL-3 (dotted line).

**Figure 3: Inhibition of apoptosis with Bcl-2 is required for development of lymphoid but not myeloid leukemias.** (A) Kaplan-Meier survival analysis of bone marrow transplants using MSCV-Bcl2, MSCV-Myc, and MSCV-Myc+Bcl2 using wild-type Balb/c donors and recipients. (B) Flow cytometric analysis of bone marrow cells from transplanted animals. Samples from MSCV-Myc and MSCV-Myc+Bcl2 mice were isolated from moribund animals. MSCV-Bcl2 mice never appeared ill and were sacrificed as controls at 5 months following marrow transplantation. Compared with MSCV-Bcl2 mice, both MSCV-Myc and MSCV-Myc+Bcl2 have a large Gr-1+/Mac1+ blast population. MSCV-Myc mice also show a population of cells pseudo-positive for both B220 and CD3. These cells were judged apoptotic cells as judged by Annexin V and 7AAD staining, morphology and failure to give disease in secondary transplants (not shown). (C) Flow cytometric analysis of thymocytes from the same leukemic and control animals. MSCV-Myc+Bcl2 mice show an increase in B220+IgM- early B cells in the thymus while MSCV-Myc mice show a relative loss of mature single positive thymocytes. (D) and (E) Flow cytometric analysis of lymph node cells isolated from MSCV-Myc+Bcl2, MSCV-Myc, and MSCV-Bcl2 animals. Both MSCV-Bcl2 and MSCV-Myc mice have a near-normal complement of B and T cells (D) Immunophenotype of lymph node cells of transplanted mice. B220-positive cells predominate in MSCV-Myc+Bcl2 mice at the expense of CD3 positive T cells. (E) Lymph node cells in MSCV-Myc+Bcl2 mice are
immature B lymphoblasts (CD43+, IgM-). In contrast, lymph node cells from MSCV-Myc and MSCV-Bcl2 mice are a mixture of mature B cells (CD43-, IgM+) and mature T cells (CD4 and CD8 single positive cells). (F) Histopathologic analysis of hematopoietic tissues from MSCV-Myc+Bcl2, MSCV-Myc and MSCV-Bcl2 mice. MSCV-Myc+Bcl2 mice have myeloid blast cells in the bone marrow and lymphoid blast cells in the lymph node and thymus. MSCV-Myc mice have myeloid blast cells in the bone marrow, mostly mature lymphocytes in the lymph nodes, with some invading myeloid blasts, and normal appearing thymocytes in the thymus. MSCV-Bcl2 mice have mature appearing, apparently normal cells in the bone marrow, lymph nodes and thymus.

Figure 4: Malignant cells from MSCV-Myc and MSCV-Myc+Bcl-2 mice are readily transplantable into secondary recipients. Kaplan-Meier survival analysis of secondary recipients of (A) MSCV-Myc and (B) MSCV-Myc+Bcl2 cells. Viable mononuclear cells isolated from the spleens of moribund primary animals were counted, serially diluted and injected into sub-lethally irradiated syngeneic mice. Number of mononuclear cells injected per mouse is shown. Comparison of A and B shows a higher frequency of leukemia-initiating cells in MSCV-Myc+Bcl2 mice.

Figure 5. Myc-induced myeloid leukemias are characterized by increased bone marrow apoptosis. (A) Apoptotic cells in the bone marrow of moribund leukemic mice was assessed by annexin V and 7-AAD staining and followed by flow cytometry. MSCV-Myc+Bcl2 (MiB)→Balb/c showed 15% annexinV and/or 7-AAD positive cells compared with 38% dying cells in matched stain mice expressing Myc but not Bcl-2.
Bone marrow from leukemic Myc→Ink4a−/− mice are 5% apoptotic compared with 29% apoptotic cells in the marrow of wild-type mice expressing Myc (Myc→Ink4a+/−, bottom panels). A representative of three independent analyses is shown. (B) Bone marrow sections from moribund leukemic mice were prepared and stained with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). Myc induces AML and apoptosis, while Myc+Bcl2 induces AML and ALL. Expressing Myc in bone marrow with targeted disruption of Ink4a gene (Ink4a−/−) yield results similar to Myc+Bcl2. MiB, MSCV-Myc+Bcl2; Myc, MSCV-Myc; Ink, Ink4a.

Figure 6. *MSCV-Myc* tumors display normal karyotypes and are polyclonal. (A) Spectral karyotyping of malignant spleen cells from five *MSCV-Myc* did not reveal any clonal abnormalities. A representative karyotype, 40, XX[10], is shown. (B) Analysis of proviral integration by Southern blot hybridization using an MSCV-specific (Psi region) probe. Digestion of spleen genomic DNA (gDNA) with an enzyme (XbaI) that cuts twice in the provirus releases a single 4.2kb proviral band in *MSCV-Myc* mice (arrow, lanes 2-8) demonstrating the presence of provirus in affected tissues but not in Balb/c control (lane 1). Digestion of genomic DNA with an enzyme (BglII) that cuts once in the provirus reveals polyclonal smearing and oligoclonal bands. Over 15 individual murine leukemias were analyzed and no clonal tumors were found. A representative blot is shown. Lanes 2, 3, 4, 7, 8 are from primary *MSCV-Myc→Balb/c* mice. Lane 5, and 13 are from primary *MSCV-Myc→Black6* spleens and lanes 6 and 14 are gDNA from an isolated granulocytic sarcoma. Lanes 1 and 9 are Balb/c control splenocytes.
Figure 7. *MSCV-Myc* leukemias contain intact Arf-p53 pathways.  (A) Germline DNA from spleens of leukemic mice was analyzed by Southern hybridization using an Ink4a exon 1β probe. Lanes 1 and 6 are Balb/c control mice and demonstrate the germline configuration of the locus. Arrowheads indicated size of germline fragments. Lanes 2-5 and 7-10 are *MSCV-Myc* spleen cells. Lanes 1-5 are spleen genomic DNA samples digested with XbaI and 6-10 are digested with BglII. The band in lane 6 is due to inadvertent DNA overloading noted on ethidium stained gel (data not shown). In all leukemias examined, the Ink4 locus was found to be in germline configuration.  (B) Western blot analysis of p53 protein and the p53 target p21 in isolated leukemia cells before and after γ-irradiation. Leukemia cells were purified from spleens of moribund *MSCV-Myc* mice by FACS sorting on GFP and forward/side scatter profiles. Lysates were isolated at time zero (lanes 1-3) and from parallel cell aliquots two hours after irradiation with 500Gy (lanes 4-6). All lanes have detectable p53 and the 2-hour samples reveal increased p21 levels, consistent with expected upregulation by intact p53 response. β actin levels indicated equivalent protein loading in all lanes.  (C) Western analysis of proteins from tissues of leukemic and control transplanted animals. Myc protein is expressed in all *MSCV-Myc* and *MSCV-Myc+Bcl2* mice. Bcl-2 is only expressed in *MSCV-Myc+Bcl2* mice. Bcl-XL is expressed in normal bone marrow as well as unfractionated leukemia samples due to normal cell contamination (see E).  (D) Western analysis of p19^Arf^ and Bcl-2 proteins in transplanted animals. Arf is expressed in all tumors analyzed. Arf-null mouse embryonic fibroblasts (MEF) are shown as negative control. Bcl-2 is expressed only in *MSCV-Myc+Bcl2* samples. (E) Comparison of Bel-XL protein levels in unfractionated and purified tumor cell populations. Unfractionated tumor tissues show abun-
dant Bcl-XL protein due to contamination with normal cells. Bcl-XL levels in purified leukemia cells is much lower (see panel B). (F) Comparison of c-Myc expression in $E_\mu$-Myc lymphoma and MSCV-Myc leukemia. Whole cell lysates were prepared from tumor bearing lymph node from $E_\mu$-Myc mouse, and spleen cells from MSCV-Myc (Myc), MSCV-Myc+Bcl2 (Myc+Bcl2), and normal Balb/c control (BALB/c). Expression levels appear equivalent in samples from retroviral and transgenic models.
Figure 1

A

B

Bone Marrow

Myc Ink4a−/−

Myc Ink4a+/−

GFP Ink4a−/−

GFP Ink4a+/−

C

Lymph Node

Myc Ink4a−/−

Myc Ink4a+/−

GFP Ink4a−/−

GFP Ink4a+/−

D

Lymph Node

Myc Ink4a−/−

Myc Ink4a+/−

GFP Ink4a−/−

GFP Ink4a+/−

E

BM

LN

Thy

Myc Ink4a−/−

Myc Ink4a+/−

GFP Ink4a−/−

GFP Ink4a+/−
Figure 2

A

MIG  
\[ \text{X} \text{\overline{X}} \text{\overline{X}} \]
\[ \text{C-MYC} \text{ IRES GFP} \]

Bcl2  
\[ \text{BCL-2 IRES GFP} \]

MIB  
\[ \text{C-MYC} \text{ IRES BCL-2} \]

GFP  
\[ \text{IRES GFP} \]

B

3T3  Myc  Bcl2  Myc+Bcl2  GFP

c-Myc
Bcl2
\[ \beta\text{-Actin} \]

1  2  3  4  5

C

Number of cells (x10^4/ml)

Days after withdrawal of IL-3
Figure 3

A

Cum. Survival vs. Days post transplantation for different conditions.

B

Flow cytometry analysis showing Gr-1, Mac-1, B220, CD3, CD4, and IgM expression levels.

C

Comparison of Myc, Bcl2, and Myc+Bcl2 conditions in terms of B220, CD3, CD4, CD8, and IgM expression levels.

D

Histograms for Gr-1, Mac-1, B220, CD3, CD4, and IgM expression levels.

E

Comparison of Myc, Bcl2, and Myc+Bcl2 conditions in terms of B220, CD3, CD4, and CD8 expression levels.

F

Histological images showing BM, LN, Thy, Myc+Bcl2, Myc, and Bcl2 conditions.
Figure 4

A

B

Cum. Survival

Days post transplantation

Cum. Survival

Days post transplantation

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Figure 5
Figure 6
Figure 7

A

B

C

D

E

F
c-Myc rapidly induces acute myeloid leukemia in mice without evidence of lymphoma-associated anti-apoptotic mutations

Hui Luo, Qing Li, Julie O'Neal, Friederike Kreisel, Michelle M Le Beau and Michael H Tomasson