Overexpression of N-Myc Rapidly Causes Acute Myeloid Leukemia in Mice

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Abstract

N-MYC encodes a basic helix-loop-helix/leucine zipper (bHLH/LZ) transcription factor that is frequently overexpressed in human neuroblastoma. N-MYC overexpression has also been reported in human acute myeloid leukemias (AML), which we show here is a frequent event. Myeloid cells in N-Myc–overexpressing mouse bone marrow hyperproliferate but those in c-Myc–overexpressing bone marrow do not. The NH2-terminal transactivation domain, nuclear localization signal, and bHLH/LZ domain of N-Myc are essential for this effect. Microarray analysis revealed 969 differentially expressed genes between N-Myc– and c-Myc–overexpressing myeloid cells. N-Myc–overexpressing cells showed decreased transforming growth factor β signaling and increased c-Jun-NH2-kinase signaling, both of which are associated with proliferation and leukemogenic transformation of myeloid cells. Mice transplanted with bone marrow expressing wild-type N-Myc developed clonal and transplantable AML after ~1 month; those transplanted with bone marrow expressing mutant N-Myc did not. Twist, a known suppressor of the p19Arf/p53 pathway, was up-regulated in all tumors. These results show that N-Myc overexpression is highly oncogenic in mouse myeloid cells and suggest that N-MYC up-regulation contributes to human myeloid leukemogenesis. [Cancer Res 2007;67(22):10677–85]

Introduction

N-MYC was originally identified as a gene amplified in human neuroblastoma cells that shows high homology with c-MYC (1, 2). N-MYC encodes a 64-kDa nuclear phosphoprotein that is a transcriptional activator/repressor (3). Like other Myc family members, N-Myc harbors a highly conserved NH2-terminal transactivation/repression domain containing two Myc boxes (4). Its COOH-terminal basic helix–loop–helix/leucine zipper (bHLH/LZ) domain forms a heterodimeric complex with the bHLH/LZ protein Max (5), which mediates DNA binding to cis-DNA sequences called the E-box (CACGTG; refs. 5, 6) in the promoter/enhancer regions of elements (YYCAYYYYY) and cause transrepression (7). The E-box (CACGTG; refs. 5, 6) in the promoter/enhancer regions of neuroblastoma, retinoblastoma, Wilms tumor, and small-cell lung carcinomas (8, 9). The oncogenic properties of N-Myc have been studied in vivo in a transgenic mouse model of neuroblastoma in which N-Myc is expressed under the control of the tyrosine hydroxylase gene promoter (10, 11). However, the mechanisms through which N-Myc mediates tumorigenesis remain unknown.

N-Myc is also involved in hematopoietic malignancies. For example, the N-Myc locus is a common target of retroviral integration in mouse T-cell lymphoma (12) and both Epi-c-Myc and Epi-N-Myc transgenic mice develop B-cell lymphoma (13). Hirvonen et al. (14, 15) reported that N-Myc expression was elevated in five of six human primary acute myeloid leukemia (AML) samples, whereas it was expressed in one of nine acute lymphocytic leukemia cases. We reported that expression of N-Myc was elevated in two of two human AML cases with t(12;22)(p13;q11) expressing the MN1-TEL fusion oncoprotein and HOX9, a homeobox protein frequently overexpressed in human AML (16). A mouse model for t(12;22) leukemia expressing MN1-TEL and HOX9 developed AML with elevated N-Myc expression. These data suggest that N-Myc plays a role in the etiology of AML. To address this possibility, we investigated the frequency of N-Myc overexpression in human AML. We also transplanted hematopoietic cells transduced with N-Myc retroviruses into wild-type mice to determine whether AML would develop.

Materials and Methods

Quantitative reverse transcription-PCR of pediatric AML samples. All patient materials used in this article have been described previously (17). Informed consent for the use of the leukemic cells was obtained in accordance with the Declaration of Helsinki, and study approval was obtained from the St. Jude Children’s Research Hospital Institutional Review Board.

Real-time reverse transcription-PCR (RT-PCR) was performed using a 7900HT sequence detection system. Briefly, the cDNA of patient bone marrow RNA was synthesized with 50 ng total RNA. CD34+ bone marrow cells from a healthy donor (StemCell Technologies) was synthesized with 500 ng total RNA. Both were done in a 20 μl total volume using Taq Man Reverse Transcription Reagent (Applied Biosystems) following the manufacturer’s recommendations, and one tenth of the cDNA sample was used for a PCR reaction. As an internal control, amplification of hypoxantine phosphoribosyltransferase (Applied Biosystems) was performed in the same reaction mix and detected with an alternatively labeled probe. Duplicates of each standard and sample were assessed. N-Myc primer sequences are available upon request.

Vector constructs. Hemagglutinin (HA)–tagged wild-type N-Myc cDNA was cloned into the EcoRI site of the mouse stem cell virus–internal ribosome entry site–yellow fluorescent protein (MSCV-RES-YFP) retroviral vector. HA-tagged mutant N-Myc cDNAs (Fig. 1; Supplementary Data) lacking sequences encoding the NH2-terminal transactivation/repression domain [amino acid (aa) 13–194; the first aa of wild-type N-Myc was designated as aa 1], or the nuclear localization signal (NLS; aa 273–367), or the COOH-terminal bHLH/LZ domain (aa 380–455) were generated by PCR and cloned into the same EcoRI site 5' of the IRES sequence of

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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MSCV-IRES-YFP, generating MSCV-N-Myc-IRES-YFP (Mock) virus or empty MSCV-IRES-YFP (Mock) virus were inoculated in liquid cultures in the presence of SCF (50 ng/mL), IL-3 (20 ng/mL), and IL-6 (30 ng/mL). Numbers of viable cells are plotted. Bone marrow cells transduced with MSCV-IRES-YFP containing N-Myc (●) or no insert (○). Experiments repeated in triplicate using different cells yielded identical results. Points, mean; bars, SE. Inset, expression of N-Myc protein in bone marrow cells transduced with MSCV-N-Myc-IRES-YFP or MSCV-IRES-YFP retrovirus. N-Myc protein present in YFP-sorted cells was detected by immunoblotting using an anti-HA antibody. GAPDH detection was used as a loading control. Expression of Cdk4 in the same cells on a similar Western blot using an anti-Cdk4 antibody. GAPDH detection was used as a loading control. B, May-Giemsa staining of N-Myc–overexpressing cells in culture (top). Black arrowhead, a blast-like cell; red arrow, a partially differentiated cell with a ring-shaped nucleus. Bottom, flow cytometry surface marker analysis of the N-Myc–overexpressing cells. C, cell cycle analysis of N-Myc– or c-MYC–overexpressing bone marrow cells. Cell cycle profile of YFP-sorted bone marrow cells transduced with N-Myc-IRES-YFP, c-MYC-IRES-YFP, or empty virus was analyzed by flow cytometry of propidium iodide–stained nuclei. *, P < 0.05, significant differences between N-Myc– or c-MYC–overexpressing cells and empty virus. D, YFP-sorted bone marrow cells transduced with N-Myc retroviruses or empty retrovirus were plated in semisolid culture (MC-1) in the presence of SCF (50 ng/mL), IL-3 (10 ng/mL), IL-6 (10 ng/mL), and erythropoietin (3 units/mL). Numbers of myeloid colonies (CFC) were scored after 8 d of culture. CFCs harvested from the MC-1 (or MC-2) were seeded into MC-2 (or MC-3) in the presence of the same cytokines. Columns, mean (n = 9); bars, SE. Inset, May-Giemsa staining of N-Myc–overexpressing CFCs. Black arrows, blast cells; red arrow, a committed myeloid cell with a ring-shaped nucleus.

Hematopoietic progenitor assays. Methylcellulose-based culture (MC) of bone marrow cells was performed using MethoCult GFM3434 (StemCell Technologies) containing mouse SCF (50 ng/mL), mouse IL-3 (10 ng/mL), human IL-6 (10 ng/mL), and human erythropoietin (3 units/mL). Colonies were counted after 8 days of culture. For serial replating assays, cells were harvested from the MC-1 (or MC-2) after 8 days of culture and replated in MC-2 (or MC-3) using GFM3434.

Analysis of cell proliferation and viability. Cells were grown in liquid culture in IMDM supplemented with 20% FCS, in the presence or absence of SCF (50 ng/mL), IL-3 (10 ng/mL), and IL-6 (30 ng/mL). Numbers of viable cells and dead cells were determined by trypan blue dye exclusion.

Bone marrow transplantation. Retrovirally transduced bone marrow cells (0.5 × 10^6) were injected i.v. into lethally irradiated (9.5 Gy) syngeneic recipients. Tumor cells (1 × 10^6) of diseased primary recipients were transplanted into lethally irradiated recipients or used for sorting and cell culture experiments.
transplanted into sublethally irradiated (7.5 Gy) syngeneic secondary recipients. Mice were monitored daily and euthanized per our institutional protocol when moribund.

**Flow cytometric analysis and cell sorting.** Expression of cell surface markers and YFP was determined by flow cytometry (FACS-Calibur or LSRII; BD Immunocytometry Systems) as described (16). Cell cycle analysis was performed by flow cytometry and ModFit software (Vegeti Software House) of propidium iodide–stained nuclei as described previously (18). The apoptotic index of the cells was determined by flow cytometry using Annexin V/propidium iodide staining as described previously (18). Cells were sorted by a fluorescence-activated cell sorter (FACS; FACSAria or FACSVantage SE; BD Immunocytometry Systems).

**Sorting mouse bone marrow into stem cell fractions.** Following the methods of Akashi et al. (19), we isolated mouse bone marrow progenitors representing hematopoietic stem cells (HSC), myeloid/erythroid progenitors (MEP), common myeloid progenitor (CMP), granulocyte/monocyte progenitor (GMP), and common lymphoid progenitors (CLP). Their identities were verified by RT-PCR analysis of signature genes (19).

**Western blot analysis.** Western blots were performed using HA (3F10; Roche Applied Science), c-MYC (OP101f; Calbiochem), Cdk4 (c-22; Santa Cruz Biotechnology), cyclin D1 (72-13G; Santa Cruz Biotechnology), cyclin D2 (c-17; Santa Cruz Biotechnology), E2F1 (KHF5; Zymed Laboratories), Bcl-2 (3F11; BD PharMingen), Bcl-X1 (4; Transduction Laboratories), Twist (c-17; Santa Cruz Biotech), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; c-11; Santa Cruz Biotechnology) antibodies.

**Histologic analysis.** Paraffin-embedded tissue sections were stained with H&E, and cytospin slides were stained with May-Giemsa. Images of tissue sections and cytospins were obtained using a BX-50 microscope (equipped with a UPlanFL; ×40/0.75 or ×100/1.30 numerical aperture objective; Olympus) with a SPOT camera and SPOT Advanced imaging software (Diagnostic Instruments). The original magnification for tissue sections was ×400 and ×1,000 for cytospins. Images of colonies were obtained using a Gel Doc EQ System (Bio-Rad). Images of mice were obtained using a DiMAGE digital camera (Konica Minolta).

**Clonality analysis.** The clonality of tumor cells was determined by hybridization of a Southern blot containing EcoRI-digested genomic DNA with an IRES-YFP probe.

**Microarray analysis.** Lin− cells were sorted from pooled bone marrow of five 12-week-old C57Bl/6 mice. After overnight culture in SCF/IL3/IL6, the cells were transduced with MSCV-IRES-YFP, MSCV-c-MYC-IRES-YFP, or MSCV-N-Myc-IRES-YFP for 2 days and then Mac1+/Gr1+/YFP+ cells were sorted by FACS. RNA was isolated using TRIzol at day 5 after bone marrow isolation, and microarray analysis was performed using the Affymetrix GeneChip Mouse Genome 430 2.0 array and GeneSpring version 7.2 software (Agilent Technologies) as described (20). Genes were normalized (to 50 percentile of the chip and to median of each gene), and data were filtered using the following criteria: (a) they had to have a “present” or “marginal” call in more than three samples, and (b) changes in N-Myc or c-MYC expression in samples had to be >2-fold compared with expression in control samples. The filtered genes were clustered by K-means using standard correlation to find those that were up-regulated or down-regulated (>2-fold) specifically in N-Myc or c-MYC samples. Significance of the fold change in expression was tested post hoc, and genes showing significant difference (P < 0.05) were subjected to hierarchical clustering using standard correlation. Changes in gene expression were analyzed using Ingenuity Systems software to identify significant differences in biological pathways in Mac1+/Gr1+/YFP+ cells expressing N-Myc, c-MYC, or empty vector.

**Statistical analysis.** Survival analysis was done using the Kaplan-Meier method and log-rank test. Statistical comparison of two independent groups was done by Student's t test. Statistical significance was assigned when the probability that there was no difference between two variables was <0.05.

### Results

**NMYC overexpression in pediatric AML.** To assess the incidence of N-MYC overexpression in human AML, we queried the expression profiles of blast cells from 137 pediatric patients with AML treated at St. Jude Children’s Research Hospital. Those gene expression profiles have been reported previously (17). N-MYC expression levels in AML samples from patients with favorable, intermediate, or unfavorable prognoses were compared with that in CD34+ cells from four healthy bone marrow donors (Table 1). Depending on the AML subtype, 24% to 40% of the samples expressed at least 2-fold more N-MYC than did normal bone marrow. RNA from 37 patient samples was available, and by using quantitative RT-PCR (qRT-PCR), we redetermined their level of N-MYC expression relative to that in normal CD34+ bone marrow cells (Table 1). Depending on the AML subtype, 20% to 100% of samples expressed N-MYC between 2- and 33-fold higher than normal bone marrow (Supplementary Fig. S2). These analyses showed that N-MYC is frequently up-regulated in pediatric AML.

N-Myc–mediated enhanced proliferation of myeloid progenitors depends on the N-terminus domain, NLS, and bHLH/LZ domain of N-Myc. Previously, we reported N-Myc up-regulation in AML cells of mice expressing the MNI-TEL fusion protein and overexpressing HOXA9 (16). To determine whether overexpression of N-Myc affects the proliferation of hematopoietic cells, we transduced mouse bone marrow cells with a bicistronic MSCV-N-Myc-IRES-YFP or MSCV-IRES-YFP (mock) retrovirus. Expression of N-Myc in YFP+–sorted cells was confirmed by Western blot (Fig. 1A). To determine their growth characteristics, sorted YFP+ cells were inoculated in liquid culture in the presence of cytokines SCF, IL-3, and IL-6. After 96 h, the N-Myc–overexpressing culture contained 6-fold more viable cells than did the control culture (Fig. 1A). The cells had myeloid morphology (i.e., blast cells and cells with ring-shaped nuclei) and expressed myeloid-specific markers Mac1 and Gr1 (Fig. 1B) but not B- or T-lymphoid markers B220 and CD3 (data not shown). Therefore, N-Myc stimulated proliferation of myeloid cells under this culture condition.

### Cell cycle status of the culture was determined by propidium ioxide staining of cell nuclei, and DNA content was determined by

**Table 1. N-MYC expression in pediatric AML samples**

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<th>Subgroup (karyotypic abnormality)</th>
<th>Affymetrix arrays</th>
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<td>Array average* (%)</td>
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<td>Other</td>
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*Percentage of patient samples expressing N-MYC 2-fold or higher than normal CD34+ cells (four donors), as determined by two Affymetrix probes.

†Percentage of patient samples expressing N-MYC 2-fold or higher than normal CD34+ cells (one donor), as determined by qRT-PCR.
flow cytometry analysis. Consistent with the proliferation assay, the percentage of cells in S phase or G2-M phase was significantly higher in cultures overexpressing N-Myc than in controls, whereas the percentage in G0-G1 phase was substantially lower than that in controls (Fig. 1C), suggesting that N-Myc stimulates cell cycle traverse. Western blot analysis showed up-regulation of Cdk4 in N-Myc–overexpressing cells (Fig. 1A), whereas the expression of cell cycle regulators cyclin D1, cyclin D2, and E2F1 was unchanged (data not shown).

To elucidate the structure-function relations of the N-Myc proliferation–stimulating effect in myeloid cells, we repeated our experiments using retroviruses encoding N-Myc mutants lacking the NH2-terminal transactivation/repression, the NLS, or the bHLH/LZ domain (Supplementary Fig. S1). Although the efficiency of transduction and level of expression of mutant N-Myc viruses in the bone marrow were similar to that of wild-type N-Myc virus (data not shown), the mutants did not stimulate myeloid cell proliferation or increase the percentage of cells in S phase or G2-M phase. Therefore, each functional domain of N-Myc is probably essential for stimulating myeloid proliferation.

**N-Myc enhances the repopulating activity of myeloid progenitors.** To further study the effect of N-Myc expression on myeloid progenitors, we plated YFP-sorted cells transduced with MSCV-N-Myc-IRES-YFP or control MSCV-IRES-YFP retrovirus in MCs in the presence of SCF, IL-3, IL-6, and erythropoietin. During initial culture (MC-1), N-Myc–transduced cells produced almost 4-fold more colonies than did controls; thus, N-Myc overexpression increased the number of progenitors during the 72-h transduction process (Fig. 1E). Also, the size of the N-Myc–overexpressing colonies was larger than that of controls (data not shown), which was consistent with their growth in liquid culture (Fig. 1A).

To analyze the repopulation capacity of these cells, we performed serial MC replating assays as described previously (16). After MC-1, cells were collected and replated either once (MC-2) or twice (MC-3) in the presence of the same cytokines. During MC-2 and MC-3, cells continued to produce significantly more and larger colonies than did controls, suggesting that N-Myc enhances the repopulating ability of hematopoietic progenitors and stimulates their proliferation in the presence of cytokines (Fig. 1D). N-Myc–overexpressing cells recovered from an MC-2 assay appeared blast-like and had large nuclei and scant cytoplasm; some cells showed myeloid differentiation featuring a ring-shaped nucleus (Fig. 1D). Flow cytometry showed that both the primary and secondary colony-forming cells (CFC) were YFP+, confirming that they expressed N-Myc, Mac1+ and Gr1+ but not B220, CD3, or Ter119 (data not shown). In addition, the cells could be replated to at least MC-10. MC-3 cells readily generated suspension cultures, which could be maintained for at least 12 weeks in the presence of IL-3 without a phenotypic change occurring (data not shown). This further supported the notion that N-Myc strongly stimulates the self-renewal capacity of myeloid progenitors, whereas none of the N-Myc mutants stimulated self-renewal activity of transduced bone marrow cells (data not shown).

**Figure 2.** Overexpression of c-MYC does not enhance the repopulating ability of myeloid progenitors. **A,** the c-Myc protein in YFP-sorted cells of bone marrow transduced with MSCV-c-MYC-IRES-YFP virus or empty virus was detected with anti-c-Myc antibody (inset). Fast green staining was used as a loading control. Serial replating assays of c-MYC–overexpressing cells. YFP-sorted bone marrow cells transduced with MSCV-c-MYC-IRES-YFP virus or empty virus were serially replated in MC cultures. Columns, mean (n = 3); bars, SE. Flow cytometry analysis of Annexin V/propidium iodide (PI)–stained (top) and Mac1+/Gr1+–stained (bottom) YFP+ c-Myc (c-MYC) and YFP+ vector (Vector) transduced bone marrow cells 4 d after transduction. Overexpression of c-Myc induces apoptosis and suppresses 5-fold the formation of Gr1+/Mac1+ cells. C, the difference in gene expression profiles between FACS-sorted YFP+/Gr1+/Mac1+ N-Myc–overexpressing, c-Myc–overexpressing, and vector-transduced bone marrow cells. Genes up-regulated or down-regulated (≥2-fold difference from controls; P < 0.05) in vector, N-Myc, or c-Myc samples are shown in a gene tree. Expression levels: red, high; shaded, intermediate; green, low. **Group 1,** genes up-regulated in N-Myc–overexpressing cells. **Group 2,** genes down-regulated in c-Myc–overexpressing cells. **Group 3,** genes up-regulated in c-Myc–overexpressing cells. **Group 4,** genes down-regulated in N-Myc–overexpressing cells. The number of genes in each group is indicated.
Overexpression of c-Myc represses the self-renewal capability of HSCs (21). Therefore, we also examined its effect on that of myeloid progenitors by using the same MC serial replating assay. Lin+ bone marrow cells were transduced with MSCV-c-MYC-IRES-YFP or MSCV-IRES-YFP (mock) virus, and the YFP-sorted cells were plated in MC in the presence of the cytokines used in the N-Myc experiments. Expression of c-Myc was confirmed by Western blotting (Fig. 2A). Unlike N-Myc, c-MYC overexpression increased the number of CFCs in the MC-1 and MC-2 only 1.5-fold (Fig. 2A), suggesting that c-MYC does not, or very minimally, stimulates the colony-forming activity of myeloid progenitors. In addition, c-MYC overexpression induced a 6-fold higher rate of apoptosis and suppressed the formation of Gr1+/Mac1+ cells 3-fold (Fig. 2B). The c-MYC–transduced bone marrow cells proliferated faster than empty vector–transduced bone marrow cells (Fig. 1C), but the inoculated suspension cultures stopped growing after 4 weeks (data not shown).

Because N-Myc and c-MYC are transcription factors, we believe that their functional difference resides in the differential expression of crucial target genes. Using Affymetrix microarrays, we compared gene expression profiles of YFP-sorted Mac1+/Gr1− bone marrow cells transduced with N-Myc, c-MYC, or empty vector retrovirus. Genes showing >2-fold change in expression (compared with control; P < 0.05) in the cells overexpressing N-Myc or c-MYC were clustered in a hierarchical gene tree (Fig. 2C; Supplementary Tables S1–S4). A total of 969 genes were differentially expressed in N-Myc, compared with c-MYC–overexpressing Mac1+/Gr1− myeloid cells, a number too large to pinpoint genes crucial for mediating the functional difference between N-Myc and c-MYC. Nonetheless, we identified decreased transforming growth factor β (TGFβ) signaling (up-regulation of Smad7 and down-regulation of TGFβ) and increased c-Jun-NH2-kinase (JNK) signaling in N-Myc–overexpressing cells—two phenomena associated with proliferation and leukemic transformation of myeloid cells (22–28).

We also determined the level of N-Myc and c-MYC expression in mouse bone marrow progenitor fractions by using qRT-PCR. We then purified the following bone marrow fractions by FACS: HSCs, MEPs, CMPs, GMPs, and CLPs. Compared with total bone marrow, all fractions contained variable but elevated levels of c-Myc RNA, and all but the GMP fraction contained elevated levels of N-Myc RNA (Fig. 3), suggesting that during myeloid differentiation, N-Myc expression is specifically down-regulated in GMPs.

The growth-stimulating effect of N-Myc is IL-3 dependent and N-Myc promotes apoptotic cell death without IL-3. To test whether the growth of cells overexpressing N-Myc depended on hematopoietic cytokines, we inoculated YFP-sorted hematopoietic cells transduced with MSCV-N-Myc-IRES-YFP or control MSCV-IRES-YFP retrovirus in suspension cultures in the presence of IL-3 and/or SCF. N-Myc–induced proliferation was exclusively IL-3 dependent, whereas c-Myc–induced proliferation was IL-3 dependent. N-Myc–induced cell death was not cytokine dependent, whereas c-Myc–induced cell death was IL-3 dependent (Fig. 4A).

Figure 3. Expression of N-Myc is down-regulated in the granulocyte/monocyte progenitor population. Mouse bone marrow (BM) fractions representing HSCs, CMPs, GMPs, MEPs, and CLPs were purified by FACS, and the amount of N-Myc and c-Myc RNA in these fractions was determined by qRT-PCR relative to GAPDH expression. N-Myc mRNA is specifically down-regulated in the GMP.

Figure 4. Overexpression of N-Myc enhances the rate of apoptosis of myeloid cells in the absence of IL-3. A, cytokine-dependent growth of N-Myc–overexpressing cells. Bone marrow cells transduced with N-Myc, c-MYC, or empty vector retrovirus whether the growth of cells overexpressing N-Myc depended on hematopoietic cytokines, we inoculated YFP-sorted hematopoietic cells transduced with MSCV-N-Myc-IRES-YFP or control MSCV-IRES-YFP retrovirus in suspension cultures in the presence of IL-3 and/or SCF. N-Myc–induced proliferation was exclusively IL-3 dependent, whereas c-Myc–induced proliferation was IL-3 dependent. N-Myc–induced cell death was not cytokine dependent, whereas c-Myc–induced cell death was IL-3 dependent (Fig. 4A).
in vivo whether N-Myc overexpression in bone marrow causes malignancy (31), and its overexpression may further increase Bid efficiency and expression level of wild-type or mutant N-Myc were each into lethally irradiated syngeneic recipients. The transduction

Overexpression of c-MYC stimulates proliferation and apoptosis (29). Therefore, we examined whether N-Myc overexpression would enhance apoptosis of myeloid cells by culturing the YFP-sorted cells without added cytokines. A cell survival assay using trypan blue dye exclusion revealed that 75% of the N-Myc–overexpressing cells had died within 24 h of cytokine withdrawal; only 30% of the control cells had died (Fig. 4B). The percentage of apoptotic cells, as determined by flow cytometry (Annexin V+ (propidium iodide) after 16 h of cytokine withdrawal, was 4-fold higher in the N-Myc–overexpressing cells than in control cells (Fig. 4C). Together, these data suggested that similar to c-MYC in pre-B cells, N-Myc stimulates proliferation and apoptosis of myeloid cells.

Western blot analysis showed down-regulation of the antiapoptotic proteins Bcl-2 and Bcl-XL in the N-Myc–overexpressing cells (Fig. 4D). This finding is consistent with previous data showing that c-MYC overexpression induces down-regulation of these proteins in pre-B cells (30). In addition, expression profiling showed that N-Myc–overexpressing YFP+/Gr1+/Mac1+ cells up-regulated Bid and caspase8, which encode proteins involved in death receptor–mediated apoptosis. Bid plays an important role in myeloid cell homeostasis (31), and its overexpression may further increase apoptosis of IL3-deprived N-Myc–overexpressing cells.

N-Myc overexpression causes AML in vivo. To determine whether N-Myc overexpression in bone marrow causes malignancy in vivo, we transduced mouse Lin– cells with MSCV-N-Myc-ires-YFP (n = 11), MSCV-ΔN-N-Myc-ires-YFP (n = 7), MSCV-ΔNLS-N-Myc-ires-YFP (n = 7), MSCV-ΔhHLH/LZ-N-Myc-ires-YFP (n = 6), or control MSCV-IRES-YFP (n = 5) retrovirus, and transplanted each into lethally irradiated syngeneic recipients. The transduction efficiency and expression level of wild-type or mutant N-Myc were the same, as monitored by flow cytometry analysis of YFP expression. All recipients of bone marrow transduced with N-Myc retrovirus developed myeloid disease and died within 50 days after transplantation, whereas mice transplanted with bone marrow transduced with N-Myc mutants or empty virus all survived (Fig. 5A). All sick mice showed a markedly enlarged spleen and liver (Fig. 5B), increased numbers of peripheral WBC (5.2 × 10^9 ± 12.4 × 10^9/mL; range, 16.9 × 10^9–224.2 × 10^9/mL; n = 9), mild anemia (hemoglobin, 10.2 ± 0.56 g/dL; range, 6.6–12.4 g/dL; n = 9), and thrombocytopenia (367.1 ± 7.1 × 10^9/L; range, 152 × 10^9–836 × 10^9/mL; n = 9). In these mice, the bone marrow consisted almost exclusively of N-Myc–cYFP+ myeloid blasts (Fig. 5B) featuring a large cleaved or ring-shaped nucleus with scant cytoplasm. The percentage of blasts in the bone marrow was 90.2 ± 3.1% (n = 5), which satisfied the criteria for AML (Bethesda proposal; ref. 32). Leukemic cells extensively infiltrated organs, such as liver, spleen, and lung (Fig. 5B), and expressed N-Myc and myeloid-specific markers (Mac1, Gr1) but not lymphoid-specific markers (CD3, B220; Fig. 5C). Analysis of the cells for expression of more differentiated markers (CD115, monocytes; F4/80, macrophages; CD88, granulocytes) showed that on average, few cells differentiated beyond the Gr1+Mac1+ stage (with low variability between tumors; CD115, 0.3%; F4/80, 8.4%; CD88, 4.5%).

Southern blot analysis with an Ires-YFP probe showed that all the AML cells were oligoclonal or monoclonal, whereas before transplantation the N-Myc–transduced bone marrow cells were polyclonal (Fig. 6A). In addition, the leukemic cells were readily transplantable into syngeneic secondary recipients (Fig. 6B), all of which (n = 7) died within 30 days, showing essentially the same disease as the primary recipients (Fig. 6B) but exhibiting a more severe anemia (hemoglobin, 7.63 ± 1.07 g/dL; range, 3.7–9.7 g/dL; n = 6), thrombocytopenia (187.1 × 10^9 ± 54.5 × 10^9/mL; range, 118 × 10^9–405 × 10^9/mL; n = 6), and elevated WBC counts (25.3 × 10^9 ± 5.68 × 10^9/mL; range, 13.9 × 10^9–42.4 × 10^9/mL; n = 6). Comparing the clonality of the primary and secondary leukemias by Southern blot showed emergence or disappearance of unique N-Myc bands. PCMV-YFP transduced bone marrow recipients all died within 50 days (n = 6), whereas mice transplanted with bone marrow transduced with N-Myc mutants or empty virus all survived (Fig. 5A). All sick mice showed a markedly enlarged spleen and liver (Fig. 5B), increased numbers of peripheral WBC (5.2 × 10^9 ± 12.4 × 10^9/mL; range, 16.9 × 10^9–224.2 × 10^9/mL; n = 9), mild anemia (hemoglobin, 10.2 ± 0.56 g/dL; range, 6.6–12.4 g/dL; n = 9), and thrombocytopenia (367.1 ± 7.1 × 10^9/L; range, 152 × 10^9–836 × 10^9/mL; n = 9). In these mice, the bone marrow consisted almost exclusively of N-Myc–cYFP+ myeloid blasts (Fig. 5B) featuring a large cleaved or ring-shaped nucleus with scant cytoplasm. The percentage of blasts in the bone marrow was 90.2 ± 3.1% (n = 5), which satisfied the criteria for AML (Bethesda proposal; ref. 32). Leukemic cells extensively infiltrated organs, such as liver, spleen, and lung (Fig. 5B), and expressed N-Myc and myeloid-specific markers (Mac1, Gr1) but not lymphoid-specific markers (CD3, B220; Fig. 5C). Analysis of the cells for expression of more differentiated markers (CD115, monocytes; F4/80, macrophages; CD88, granulocytes) showed that on average, few cells differentiated beyond the Gr1+Mac1+ stage (with low variability between tumors; CD115, 0.3%; F4/80, 8.4%; CD88, 4.5%).
of bands underpinning the oligoclonality of the primary AML and clonal selection of the secondary leukemia (Fig. 6A). Together, these data indicate that enforced expression of N-Myc causes aggressive AML in mice and suggests that it contributes to murine MN1-TEL/HOX99 leukemogenesis (16). N-Myc–induced AML was oligoclonal; thus, despite the short latency of the disease, additional genetic or epigenetic events are probably required to fully transform myeloid progenitors.

The simultaneous stimulation of proliferation and apoptosis of myeloid cells by N-Myc is similar to that of lymphoid cells by c-MYC. Disruption of p53-dependent apoptosis is an important secondary step in c-MYC tumorigenesis (29, 33). In N-Myc–amplified neuroblastomas, up-regulation of Twist, an inhibitor of the p19Arf/p53/Mdm2 tumor-suppressor pathway, is an important cooperating tumorigenic event (34). Therefore, we evaluated the expression of Twist, Bcl-2, and Bcl-XL in N-Myc–overexpressing AML cells. Twist expression was higher in all N-Myc-AML samples (n = 10), whereas Bcl-2 and Bcl-XL expression was lower in tumors than in bone marrow cells transduced with empty vector (Fig. 6C). However, Twist expression was not up-regulated in primary bone marrow cells transduced with N-Myc retrovirus, indicating that Twist up-regulation in AML does not directly result from N-Myc overexpression but is the result of an additional genetic or epigenetic event in the tumors. These data suggest that Twist-mediated disruption of the p19Arf/p53/Mdm2 tumor-suppressor pathway is involved in the etiology of N-Myc AML in mice.

**Figure 6.** N-Myc–expressing AML cells are clonal and transplantable. A, analysis of the clonality of AML cells. Left, Southern blot containing EcoR I-digested genomic DNA of AML cells hybridized with an iRES-YFP probe. The sample number of the tumors is indicated above each lane. C, control untransduced bone marrow. Right, primary tumor cells were transplanted into sublethally irradiated syngeneic recipient mice (n = 7), and the clonality of secondary AML cells was analyzed by Southern blotting using a GFP probe. The sample numbers indicated are identical to those in the left panel. T, tumor; Pre, pretransplant N-Myc–transduced bone marrow (YFP-sorted); P, primary AML; S, secondary AML. Arrows, bands that emerged (black) or disappeared (red) in secondary AML samples. B, survival curve of secondary transplantation of AML cells. Primary tumor cells were transplanted into sublethally irradiated syngeneic recipient mice (n = 7). A survival curve was generated according to the Kaplan-Meier method. Inset, May-Grünwald staining of a bone marrow cytospin preparation obtained from a sick secondary recipient. C, representative result of a Western blot containing lysates of bone marrow cells transduced with empty retrovirus (mock BM) or N-Myc retrovirus (N-Myc BM) or tumor bone marrow (2, 3, and 4) incubated with antibodies specific for Twist, Bcl-2, or Bcl-XL, GAPDH detection was used as a loading control. The sample numbers indicate the same tumors as those in A.

**Discussion**

Up-regulation of N-Myc plays a role in the etiology of AML (14–16). Our analysis of an existing AML expression array (17) supported this notion because N-Myc overexpression was observed in 20% to 40% of pediatric patients with AML without specific correlation with three different risk groups. Reevaluation of N-Myc expression in a subgroup of 37 of these patients by qRT-PCR showed an even higher percentage (24–100%). The most likely explanation for why N-Myc overexpression in AML was not reported in two large-scale expression array analyses (17, 35) is that in those studies, gene expression profiles from different AML subtypes were compared with each other and not with normal CD34+ cells. That comparison eliminated N-Myc as a gene with a subtype-specific expression profile.

A causative role of N-Myc in AML is further supported by our observation that retrovirus-mediated overexpression of N-Myc in mouse bone marrow stimulated the proliferation and self-renewal of myeloid cells in vitro and rapidly caused AML in vivo. N-Myc enhanced the fraction of myeloid progenitors in S phase, which coincided with increased expression of the cyclin-dependent kinase Cdk4, a protein involved in cell cycle progression (36). This closely resembles induction of Cdk4 by c-Myc in human umbilical vein endothelial cells and primary human B cells. Although we did not determine whether Cdk4 is a direct target of N-Myc, the gene is a bona fide direct target of c-Myc (36). Although c-Myc stimulates short-term growth of bone marrow cells, it does not stimulate...
self-renewal activity of myeloid progenitors under our culture conditions. This is consistent with a previous report showing that c-MYC does not enhance the repopulation of myeloid progenitors (21).

N-Myc stimulates bone marrow repopulation by controlling the expression of genes that are not targets of c-MYC. Indeed, >969 genes were differentially expressed when comparing N-Myc- and c-MYC–overexpressing YFP+/Gr1+/Mac1+ myeloid cells. Ingenuity analysis pinpointed differences in pathways that may affect proliferation and self-renewal; that is, it indicated down-regulation of TGFβ and up-regulation of Smad7. This finding strongly suggests that TGFβ signaling is reduced in N-Myc–overexpressing cells. TGFβ inhibits proliferation by activating Smad4, the activity of which is inhibited by Smad7 (22). Also, up-regulation of JNK signaling might contribute to the increased self-renewal and proliferation of N-Myc–overexpressing cells (24–28). Involvement of both of these pathways in N-Myc transformation is amenable to further experimental verification.

Recently, the effects of c-Myc on myeloid cells were reported. Here, we show that these effects can also be induced by N-Myc retrovirus–transduced bone marrow (37). We and others (21, 38, 39) have not observed such results with c-MYC retrovirus–transduced bone marrow, although our experimental conditions differed from theirs. Wilson et al. (21) and Yu et al. (39) used a different retroviral vector, and Hemann et al. (38) used a MSCV-c-MYC-IRES-GFP virus but transduced fetal liver cells rather than bone marrow cells. These differences might explain why those investigators did not obtain the AML phenotype described by Luo et al. (37).

Luo et al. (37) reported that c-Myc–transduced bone marrow cells proliferate in MC without added growth factors. This was not the case in our hands. Moreover, bone marrow transduced in vitro with c-MYC cDNA expressed by the pSR-azertroval vector (40) behaved almost identical to bone marrow transduced with MSCV-c-MYC retrovirus (Fig. 2A–C; data not shown). However, transplantation of this bone marrow into lethally irradiated recipients did not cause any malignancy during a 6-month observation period.1 It should be pointed out that there are differences in the experimental conditions of Luo et al. (37) and ours. We used Lin–bone marrow cells, whereas they used mononuclear bone marrow cells, suggesting transduction of a more primitive cell population in our case. Also, the production of virus, the prestimulation conditions might have caused the differences between our results and those reported by Luo et al. (37), but this possibility has not been ruled out.

Compared with that in other bone marrow progenitor fractions, N-Myc expression in the GMP was down-regulated. We speculate that forced overexpression of N-Myc by the retroviral vector has a profound effect on the proliferation rate and self-renewal activity of GMPs, thereby stimulating the outgrowth of Mac1+/Gr1+ cells.

Despite the short latency of disease, N-Myc–overexpressing AML cells were oligoclonal or monoclonal, suggesting that overexpression of N-Myc alone is insufficient to cause full leukemic transformation and requires additional genetic events. The clonal selection observed in the secondary recipients further supports this idea. Because c-MYC overexpression induces both proliferation and apoptosis, transformation by c-MYC requires bypass of its self-induced apoptotic response, which often occurs through inactivation of the p19Arf/p53 tumor-suppressor pathway (29, 33). Similarly, N-Myc prompts both increased proliferation and apoptosis of myeloid hematopoietic cells, as shown by the increased apoptotic response after withdrawal of cytokines, most likely a result of down-regulating Bcl-2 and Bcl-XL. N-Myc might also require the elimination of the apoptotic response. Indeed, Twist expression was up-regulated in all AMLs, although Twist is not a direct target of N-Myc. Twist cooperates with N-Myc amplification in neuroblastomagenesis (34). Therefore, up-regulation of this p19Arf/p53/Mdm2 pathway suppressor is a secondary genetic or epigenetic event, the mechanism of which remains unknown. Twist-mediated suppression of the p19Arf/p53 pathway and other non–p53-dependent apoptotic pathways (41), combined with other mutations, most likely helps complete N-Myc–induced leukemogenesis.

In conclusion, N-Myc overexpression in bone marrow strongly promotes myeloid leukemogenesis in mice. Therefore, its frequent overexpression in human AML most likely indicates a contribution of N-MYC to leukemogenesis.

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