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Neuromedin U: A Myb regulated autocrine growth factor for human myeloid leukemias

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ABSTRACT

The *c-myb* proto-oncogene has been implicated in leukemogenesis, but possible mechanisms remain ill defined. To gain further insight to this process, we employed transcript profiling in K562 cells expressing a dominant negative Myb (MERT) protein. 105 potential Myb gene targets were identified. Neuromedin U (NmU), a peptide affecting calcium transport, underwent the greatest expression change (~5x decrease). To verify a linkage between *c-myb* and NmU, their mRNA levels were quantitated using real time PCR in primary acute myeloid (AML), and lymphoid (ALL) leukemias, as well as normal hematopoietic cells. *c-Myb* was elevated in AML and ALL samples, but NmU expression was increased only in AML cells. Significantly, only AML cells expressed NmU's cognate receptor, NMU1R, suggesting the presence of a novel autocrine loop. We examined this possibility in detail. Exogenous NmU "rescued" growth suppression in K562-MERT cells, and stimulated the growth of primary AML cells. siRNA "knockdown" of NmU in K562 cells arrested cell growth. Exposing Indo-1 labeled K562 cells to NmU induced an intracellular Ca^{+2} flux consistent with engagement of the NMU1R. Combined, these results suggest that NmU expression is Myb related, and that the NmU/NMU1R axis constitutes a previously unknown growth promoting autocrine loop in myeloid leukemia cells.

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INTRODUCTION

The proto-oncogene *c-myb* encodes a transcription factor, Myb, that is expressed predominantly in immature hematopoietic cells [1-3] where it plays a critical role in definitive hematopoiesis [4-6]. c-Myb transactivates its target genes by binding to a well defined consensus sequence, [PyAAC(T/G)G], referred to as the Myb responsive element [7]. Of genes known to be Myb induced, many regulate cell proliferation, differentiation, and survival including c-myc [8-12], cdc2 [13], c-kit [14-16], GATA-1 [17], mim-1[18], myeloblastin [19], and bcl-2 [20,21].

The oncogenic potential of *myb* was first observed in birds, when it was discovered that that a truncated form of the cellular proto-oncogene, called viral-myb (v-myb), was contained within the AMV and E26 leukemia viruses [22-24]. V-myb has deletions at its 5' and 3' ends which results in a protein missing a small, but apparently inconsequential portion of its DNA binding domain and virtually its entire negative regulatory domain. Loss of the negative regulatory domain is thought to contribute to the proteins transforming ability[25]. These deletions may have arisen as a result of retroviral insertional mutagenesis since the myb locus is known to undergo retroviral insertion[22]. Further, retroviral insertion of *c-myb* in chicken and mouse models produces a truncated form of the *c-myb* gene which causes B- and T-cell lymphomas [26-29] and myeloid leukemias [10,30-32], respectively.

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In humans, aberrant expression of *c-myb* has been associated with leukemia [33] as well as a number of solid tumor cancers such as colon cancer [34]. Exposing human cell lines and primary leukemic blast cells to antisense c-myb oligodeoxynucleotides inhibits their growth at doses where normal hematopoietic progenitor cells survive [35]. One explanation for this differential sensitivity is that c-Myb regulates a unique set of genes in leukemic cells that are required for survival [36].

To identify potential Myb target genes in human leukemia cells we employed a tamoxifen inducible, dominant negative Myb expression construct (MERT) that had been previously utilized with success to examine Myb function in murine T-cells [20,37-39] and murine myeloblastic cells [10]. We engineered K562 cells [40] to express MERT (K562-MERT), and then performed transcript profiling experiments comparing gene expression in the presence or absence of tamoxifen. Using this strategy, we identified 105 potential Myb gene targets including neuromedin U (NmU), which underwent the largest change observed. NmU was first isolated from porcine spinal cord extracts and named for its uterine contraction activity [41]. It is reportedly expressed in bone marrow cells [42], but its function is unknown. We report herein that *c-myb* and NmU expression are elevated in primary acute myelogenous leukemia (AML) cells, and the identification of a novel autocrine growth loop in myeloid leukemia cells formed by NmU and its cognate receptor, NMU1R.

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MATERIALS AND METHODS

Adherent and Suspension Cell Culture. 293 and K562 cells were originally obtained from the American Type Culture Collection (Manassas, VA). They were maintained in Dulbecco modified media, and RPMI 1640 respectively, supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine (2 mM), and 0.5% penicillin/streptomycin (pen/strep) antibiotics (50 units/ml and 50 μg/ml, respectively). Primary hematopoietic cells were purified by Ficoll-Hypaque sedimentation from bone marrow aspirates, pheresis specimens, and peripheral blood from normal donors (n = 6) and patients with acute lymphocytic leukemia (ALL) (n = 7) or AML (n = 11). Bone marrow aspirates, pheresates, and peripheral blood were obtained in accordance with the guidelines approved by the Institutional Review Board of the University of Pennsylvania.

Primary AML cells from four patients were cultured for 8 days in duplicate in Iscove's medium supplemented with a cytokine cocktail (100 ng/ml of stem cell factor (SCF), 6 ng/ml IL-3, and 6 ng/ml IL-6) in the absence or presence of 100 μM NmU peptide (NH2-FRVDEEFQSPFASQSRGYFLFRPRN-NH2) (Invitrogen Life Technologies). Cell cultures were examined with an inverted microscope at days 2, 4, 6, and 8. On the first day there appeared to be differences in cell numbers, the cells were counted using a hemacytometer and viability determined, in order to confirm and quantitate the visual impression of differences in cell growth.

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Colony Forming Assays. K562-MERT cells (1.5 x 10^3 cells/ml) were cultured in a methylcellulose mixture containing 10 ng GM-CSF and 20 ng IL-3, in the absence and presence of 1 μM 4-hydroxytamoxifen (4-OHT) and/or 35 μM NmU peptide. As a control, K562 cells were cultured under the same conditions. Colonies were scored on day 14 of culture. One colony was defined as a colony containing 20 or more cells. The area of K562-MERT colonies was determined using IP Lab software.

MIGR-MERT Preparation. MIGR-MERT was constructed by simultaneously subcloning the DNA binding domain (DBD) of *c-myb* as a Bgl II/SphI fragment and EnER, which is comprised of a *Drosophila* Engrailed transrepressor [En] and a modified mouse estrogen receptor (ER), as a SphI/EcoRI fragment into the Bgl II/EcoR I sites of the retroviral bisictronic vector MIGR1 (generously provided by Warren Pear, University of Pennsylvania, Philadelphia, PA). To obtain the DBD of *c-myb,* pcDNA3myb was amplified in the presence of *c-myb* primers (Forward primer: 5'-AGATCTGCCACCATGAATCGAACAGATGTGCAG-3'; Reverse primer: 5'-GCATGCATTGTAGAATTCCAGTGGTT-3') using standard PCR protocols. The 360-bp amplicon was cloned into pCR®-II-TOPO® (Invitrogen) as per the manufacturer's instructions and recovered from pCR®-II-TOPO® by a Bgl II and SphI double digest. To obtain the EnER gene fragment, pEnERSN (a generous gift from L. Wolff, National Cancer Institute, Bethesda, MD) was digested with SphI and EcoRI.

Preparation of K562-MERT Cell Line. MIGR-MERT was combined with a GP plasmid, which encodes the Gag and Pol proteins, and a VSV-G plasmid, which encodes

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the viral Env protein, at a ratio of 1:1:1.5, respectively. The plasmid mixture was co-transfected into 293 cells using the Superfect™ Transfection Reagent (Qiagen) at a ratio of 1:5 of total DNA:Superfect™ Transfection Reagent and cultured as recommended by the manufacturer. The retroviral supernatant was harvested 48 hr post-transfection and filtered (0.22 μm). K562 cells (1 x 10^6 cells/ml) were then infected with filtered retrovirus in the presence of polybrene (8 μg/ml) and centrifuged at 1,500 x g for 90 min at room temperature [43]. Afterwards, the cells were washed with 10-volumes of RPMI supplemented with 10% FBS, 1% L-glutamine, and 0.5% pen/strep and cultured. K562-MERT cells were enriched based on GFP expression using a FACSTAR flow cytometer (Becton Dickenson).

Cell Cycle Analyses. K562 and K562-MERT cells (1 x 10^5 cells/ml) were treated in the absence and presence of 1μM 4-OHT for 72 hr. Following treatment, the cells were fixed with 70% ice-cold ethanol for a minimum of 30 min at 4°C. The fixed cells were washed twice with PBS, treated with 5 μg/ml of DNase-free RNase (Roche) for 30 min at 37°C, and stained with 50 μg/ml of propidium iodide (PI) for at least 30 min at 4°C in the dark. The PI stained cells were measured in a FACScan flow cytometer using the CellQuest software (Becton Dickinson) and the data were analyzed for cell cycle distribution using the ModfitLT Mac V3 software.

Isolation of RNA and Preparation of cDNA. Total RNA was isolated from K562-MERT and primary cells from normal donors, AML, and ALL patients using the RNeasy® Mini kit (Qiagen) as per the manufacturer's instructions. The RNA was subsequently treated

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with 1 U/μg of RNase-free DNase (Roche) for 1hr at 37°C and re-purified using the RNeasy® Mini kit (Qiagen) according to the manufacturer's instructions. Total RNA (0.5 μg) was then reverse transcribed with random hexamer primers (5 ng/ml) and 25 U of MoMLV reverse transcriptase (Invitrogen) as instructed by the manufacturer.

Microarray Analysis. K562-MERT cells were cultured in the absence or presence of 1 μM tamoxifen for 72 hr. Total RNA was isolated as described above and submitted to Incyte (Incyte, Wilmington, Delaware). At Incyte, polyA selection, Cy3- and Cy5-labeling, hybridization to an Incyte manufactured microarray chip containing 10,000 genes, and analyses using GemTools™, Microsoft®Access®, and Statistica® software (StatSoft, Tulsa, OK) were completed as described[44,45].

Quantitative Analysis of c-Myb, NmU, GAPDH, and 18S by Real-time PCR. Total RNA was purified from K562-MERT cells treated in the absence and presence of 1μM 4-OHT for 72 hr and reverse transcribed as described above. The cDNA was subsequently amplified in TaqMan Universal Master Mix (Perkin-Elmer Applied Biosystems) supplemented with gene specific primers (0.5 μM) and the appropriate gene specific TaqMan probe (0.1 μM). The PCR parameters set on the iCycler (BioRad) consisted of 2 min at 50°C, 10 min at 95°C, then 40 cycles each of 15 sec at 95°C and 1 min at 60°C. The same PCR conditions were used for cDNA that was synthesized from the RNA that was purified from normal donors and patients with AML or ALL. The primers and probes for *c-myb*, NmU, GAPDH, and 18S were designed using Primer Express software (Perkin-Elmer Applied Biosystems). All of the probes contained the reporter dye 6-

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carboxyfluorescein (FAM) at the 5' end and quencher dye 6-carboxytetramethylrhodamine (TAMRA) at the 3' prime end. The sequences for the primers and probes were as follows: *c-myb* (Forward primer: 5'-TCATGGAGACAGTGCACCTGTT-3', Reverse primer: 5'-GGACGATCATGCACCTTGCT-3'; probe: 5'-AGAACACCACTCCACTCCATCTCTGCCA-3'), NmU (Forward primer: 5'-GAAGACACAGAAGTTGGGCAAGT-3', Reverse primer: 5'-CTCTTCATTCTTCTCTCATGCAGGT-3'; probe: 5'-AGCAACGGATGCACAACTGACGACA-3'), GAPDH (Forward primer: 5'-GAAGGTGAAGGTCGGAGTC-3', Reverse primer: 5'-GAAGATGGTGATGGGATTTC-3'; probe: 5'-CAAGCTTCCCGTTCTCAGCC-3'), and 18S (Forward primer: 5'-GGACATCTAAGGGCATCACAGACC-3'; Reverse primer: 5'-TGACTCAACACGGGAAACCTCAC-3'; probe: 5'-TGGCTGAACGCCACTTGTCCCTCTAA-3'). A standard curve for each amplicon was included to quantitate the expression of *c-myb*, NmU, GAPDH, and 18S in the test cDNA. The standard curve consisted of five serial dilutions of a plasmid containing either *c-myb*, NmU, GAPDH or 18S and was considered acceptable when the correlation coefficient exceeded 0.995. To quantitate the amount of each gene in a given sample, the mean number of copies from a triplicate determination for each gene in the test cDNA was normalized to the mean number of copies of 18S. These values were converted to the number of copies of a given gene in 1 μg of RNA.

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Preparation of siRNA and delivery to K562 cells. Using a plasmid containing full-length human NmU (GenBank accession #NM_006681), T7-NmU-Forward primer (5'-TAA TAC gAC TCA CTA TAC CAg TTg TgC ATC CgT TgC Tg-3'), T7-NmU-Reverse primer (5'-TAA TAC gAC TCA CTA TAC CgA ACC CTg CTg ACC TTC TTC –3'), and standard PCR protocols, we obtained the template for transcription reactions. Double stranded RNA was generated from the amplicon using Megashortscript Kit (Ambion) as recommended by the manufacturer. Following transcription, the DNA template was degraded with 10 U DNAse I (15 min, 37^0C) and double stranded RNA was digested with 5 U RNAse III (Ambion) (1 hr, 37^0C) to obtain a pool of siRNA ranging from 14 to 21bp. The siRNA pool was purified using size exclusion columns (Princeton Separations Inc.) as instructed by the manufacturer and the concentration was determined by UV-spectrometry. Fluorescent-labeled siRNAs were prepared as above by substituting UTP with Chromatide UTP (Molecular Probes) in the transcription reaction. Control and NmU siRNAs (8 μg) were delivered to K562 cells by nucleofection (Amaxa Biosystems) using Nucleofection kit V and program Q29. Using fluorescent-labeled siRNA and flow cytometry, the nucleofection efficiency was determined to be 86%.

Western Blot Analysis. Cells were lysed in 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1% IGEPAL and Mini Complete protease inhibitor cocktail as instructed by the manufacturer (Boehringer Mannheim). Protein lysates (50 μg) were fractionated by 10% SDS-PAGE, transferred electrophoretically to a PVDF membrane, and incubated with anti-NMU1R (1 μg/ml) (Alpha Diagnostic International) for 48 hr at

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4^0C using standard Western blot procedures. The negative control was HL-60 cell lysates and the positive control was peripheral leukocytes from a normal donor.

Intracellular Ca^{+2} Flux. K562 cells (1×10^7) in log-phase growth were resuspended in 1 ml of pre-warmed HBSS (25 mM HEPES, pH 7.4, 2.4 mM $CaCl_2$, 1.3 mM $MgSO_4$, 10 mM glucose, 124 mM NaCl, 5 mM KCl, and 1.24 mM KH_2PO_4) and labeled with a mixture containing 3 μM Indo-1 AM (Molecular Probes) and 4 mM Probenecid for 30 min at 37^0C in the dark. The cells were then washed four times with HBSS and resuspended in fresh HBSS. Intracellular Ca^{+2} flux of viable, Indo-1 labeled K562 cells (1×10^6 cells/ml) was measured following the addition of 300 μM NmU using a fixed alignment, 6 color, 3 laser LSR flow cytometer (Becton Dickenson). The data acquired by the LSR flow cytometer were analyzed using FlowJo software version 2.7.8. Since FL4 (397-417 nm) detects Ca^{+2} bound to Indo-1 and FL5 (480-500 nm) detects free Ca^{+2}, the data are presented as the ratio of FL5/FL4 as a function of time. Ionomycin (1 mg/ml) (Sigma-Aldrich), a calcium-specific ionophore, was added to the Indo-1 labeled K562 cells following the addition of the agonist to ensure that the K562 cells were adequately labeled with Indo-1.

Statistical Analyses. The Student t-Test was used to evaluate differences in cell number in the absence and presence of NmU and/or tamoxifen. *P* values less than 0.05 were considered significant.

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RESULTS

The Effect of MERT on Cell Growth and Cell Cycle Progression in Transduced K562 Cells. Methylcellulose colony forming assays using K562-MERT cells in the absence and presence of tamoxifen revealed marked differences. In the absence of tamoxifen, GFP^+ colonies of K562-MERT and untransduced K562 cells were comparable in size (Figure 1A and 1B). However, in the presence of tamoxifen the GFP^+ colonies of K562-MERT cells appeared smaller than those K562 cells that do not express MERT (Figure 1C and 1D). FACS analysis of K562-MERT cells in the absence and presence of tamoxifen revealed a 10-fold decrease in GFP expressing K562-MERT cells while the percent of GFP^+ K562 cells transduced with MIGR1 did not change between untreated and tamoxifen treated cells (data not shown). Taken together, these data suggest that the small GFP^+ colonies of K562-MERT cells in methylcellulose cultures are due to the inhibition of Myb activity by MERT in tamoxifen treated K562-MERT cells.

Based on these observations, we hypothesized that K562-MERT cells were undergoing cell cycle arrest and/or apoptosis in the presence of tamoxifen. In untreated and tamoxifen treated K562 cells, no change in cell cycle progression was observed (Figure 2A and 2B). However, K562-MERT cells treated with tamoxifen for three days arrested at the G_1/S transition of the cell cycle compared to the untreated K562-MERT cells, demonstrating that endogenous Myb activity in these cells was abrogated by MERT in the presence of tamoxifen (Figure 2C and 2D). To determine whether the small GFP^+ colonies of K562-MERT cells (Figure 1D) in the presence of tamoxifen were the result of

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cells undergoing apoptosis, we measured the expression of Annexin V. There was no change in Annexin V expression in untreated and tamoxifen treated K562 and K562-MERT cells (data not shown). Taken together, these data indicate that the small size of GFP^+ K562-MERT colonies in the tamoxifen treated methylcellulose cultures is most reasonably attributed to impaired cell proliferation and not apoptotic cell death.

Microarray Analysis of Gene Expression in Tamoxifen Treated K562-MERT Cells. When c-Myb activity was suppressed by MERT in our model system, only 37 of 10,000 arrayed genes increased their expression >2-fold. These genes included hemoglobin, transferrin, and platelet glycoprotein IIIa (Table 1). These genes have been reported to be associated with differentiating cells, which is consistent with the fact that c-Myb's expression decreases when hematopoietic cells progress toward terminal differentiation. Conversely, the expression of 68 genes decreased >2-fold when Myb activity was inhibited by tamoxifen in K562-MERT cells. Among the most repressed in gene expression were NmU, cdc7, and H3 histone (Table 1). These genes are, for the most part, associated with cell cycle progression and proliferation, which is also consistent with what is known of c-Myb's role in hematopoietic cell development. Given that overexpression of c-Myb has been associated with human leukemias [33], we reasoned that Myb-regulated genes involved in leukemogenesis would most likely decrease when Myb activity was inhibited. Therefore, we focused our analysis on those genes that decreased in expression following tamoxifen treatment of K562-MERT cells. Of the downregulated genes, NmU was of particular interest because its expression changed the most (4.9-fold decrease) and it has been reported to be present in bone marrow [42].

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Quantitative Real-Time PCR Analyses of c-Myb, NmU, GAPDH, and 18S Expression in K562 Cell Lines and Primary Cells from Patient and Normal Donors.

To confirm the microarray data, the expression level of candidate Myb gene targets was determined in transduced and untransduced K562 cells using quantitative real-time PCR after culturing them in the absence and presence of tamoxifen for three days. Relative to the untreated K562-MERT cells, NmU expression was decreased 2.8-fold in tamoxifen treated cells (Figure 3A), a result consistent with the microarray data (Table 1). The expression of cdc7 (a previously unidentified Myb gene target) in K562-MERT cells was quantitated by real-time PCR and found to decrease 5-fold when Myb was inactivated (data not shown), a result that was also consistent with the microarray data (Table 1). As a control, NmU expression in K562 cells, and GAPDH expression in K562-MERT cells was measured by real-time PCR in the absence and presence of tamoxifen. No significant change in the number of NmU copies in K562 cells was observed between the untreated and tamoxifen treated cells (Figure 3B), indicating that tamoxifen alone does not induce NmU gene expression. As observed in the microarray experiment, there was no change in the number of GAPDH copies in untreated and tamoxifen treated K562-MERT cells (Figure 3C).

We then examined primary cells from normal donors and patients diagnosed with either ALL or AML for the expression of *c-myb* and NmU by quantitative real-time PCR. The mean expression of human *c-myb* normalized to 18S in controls and in ALL or AML patients was 1.48×10^{-4} copies/ μg of RNA, 1.57×10^{-3} copies / μg of RNA, and 1.76 x

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10^{-1} copies/ μg of RNA, respectively (Figure 3D). When compared to normal donors, this represents a 10.6 fold increase in *c-myb* expression in ALL cells, and a remarkable 1,190-fold increase in AML patients. NmU expression in the leukemia specimens appeared to parallel c-myb expression. When normalized to 18S, NmU expression in normal donors and in patients with ALL was found to be 3.97×10^{-7} and 3.10×10^{-7} copies/μg of RNA, respectively, a difference which was not statistically different. In contrast, NmU was found to be present at 1.64×10^{-4} copies/μg of RNA in AML patients, a 413-fold increase in comparison to normals or ALL patients (Figure 3E). One explanation for these results may be a "dose phenomenon," such that induction of NmU expression requires very high levels of *c-myb* expression. Alternatively, we cannot exclude that other, myeloid tissue specific factors, may also play a role in Myb's regulation of NmU expression.

Proliferation of NmU Treated K562-MERT and Primary AML Cells. When NmU is expressed in cells, it is processed into a 25-amino acid peptide that is secreted from the cell [41] and binds to NMU1R [46]. Based on the observations described above, we reasoned that one function of NmU in hematopoietic cells might be to promote proliferation. To test this hypothesis, we first examined the colony forming ability of K562-MERT cells in methylcellulose cultures in the absence and presence of NmU and/or tamoxifen and then determined the ability of NmU to stimulate the growth of primary AML cells. In the first set of experiments, K562-MERT cells were cultured in methylcellulose in the absence or presence of NmU (Figure 4A and 4B). We observed relatively no change in the number of K562-MERT colonies between untreated and NmU treated cultures (Table 2).

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However, we did observe an increase in the total area of each K562-MERT colony in the NmU treated methylcellulose cultures compared to untreated cells (Figure 4A and 4B). The mean area of the K562-MERT colonies in untreated cultures (Figure 4A) or cultures treated with only NmU (Figure 4B) was 0.358 x 10^5 □m^2 and 1.3 x 10^5 □m^2, respectively. To further demonstrate that the larger size of the K562-MERT colonies in the NmU treated cultures compared to untreated cultures was due to a greater number of cells in the colonies, we solublized the methylcellulose cultures and counted the individual cells. Untreated K562-MERT cells yielded 1.70 $\pm$ 0.223 x 10^5 cells/ml while K562-MERT cells cultured with only NmU yielded 5.21 + 0.303 x 10^5 cells/ml, which is a 3.0-fold increase in cell number between untreated and NmU treated K562-MERT cells ($P < 0.0004$) (Table 2). A similar increase in colony area, and cell number was observed with K562 cells that were cultured only with NmU compared to untreated cells (data not shown).

In the second set of experiments, we cultured K562-MERT cells in methylcellulose with tamoxifen to inhibit endogenous Myb activity and therefore inhibit NmU expression (Figure 4C and 4D). To overcome the Myb-mediated blockade of NmU expression, we supplemented one of the tamoxifen methylcellulose cultures with NmU (Figure 4D). Tamoxifen treated K562-MERT cells yield colonies that were smaller in size then those observed in the absence of tamoxifen (Figure 4A) or in the presence of NmU (Figure 4B and 4D). The mean colony area of the K562-MERT colonies in methylcellulose cultured with tamoxifen was determined to be 0.159 x 10^5 □m^2 and the cell number was calculated to be 0.443 $\pm$ 0.138 x 10^5 (Table 2). Culturing K562-MERT cells with both

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NmU and tamoxifen resulted in a 3.9-fold increase in the mean colony area and a 2.4-fold increase in cell number ($P < 0.02$) compared to K562-MERT cells cultured with tamoxifen alone. The mean colony area of K562-MERT colonies cultured with NmU and tamoxifen was 0.625 x 10^5 μm^2 and the cell number was 1.06 $\pm$ 0.597 x 10^5 cells/ml (Table 2). K562 cells cultured in methylcellulose with tamoxifen revealed no difference in the colony area or cell number compared to untreated K562 cells (data not shown).

In the third set of experiments, primary cells from four AML patients were cultured in the absence or presence of NmU. In three of four clinical samples tested, the addition of NmU to patient cell cultures resulted in a variable, but statistically significant increase in cell proliferation compared to cells cultured in the absence of NmU (Table 3). These data are consistent with the hypothesis that NmU promotes the growth of human myeloid leukemia cells.

Silencing NmU gene expression inhibits proliferation of human myeloid leukemia cells. To provide additional support for the hypothesis that NmU functions as an autocrine growth factor in myeloid leukemias we sought to silence NmU expression in K562 cells using short interfering RNA (siRNA). NmU and control siRNA were synthesized and delivered to cells by nucleofection as detailed in the "Materials and Methods". 24 hours post-nucleofection of NmU targeted siRNA, NmU expression in K562 cells was inhibited ~70%, as measured by quantitative real-time PCR, when compared to mock nucleofected cells (Figure 5A). The expression of 18S RNA was unchanged when measured at the same time point suggesting specificity of the "knock

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down". When compared to mock, or control siRNA treated cells, viability of K562 cells nucleofected with NmU siRNA decreased ~40% ($p<0.006$) (Figure 5B). Taken together, these results are consistent with the hypothesis that NmU supports leukemic cell proliferation.

Expression of NMU1R in myeloid and lymphoid cells. To be certain that the effects of NmU observed on leukemic cell growth were infact due to engagement of NMU1R, we sought to demonstrate its presence in cells in which a biologic effect was, or was not, observed [46]. To determine the presence of NMU1R in cell lysates of K562, K562-MERT, and patients with AML or ALL, we performed Western blot analyses. Both K562 and K562-MERT cells express NMU1R (44-kDa) (Figure 6, lanes 3 and 4). In addition, K562-MERT cells appear to express a dimer form of the receptor (Figure 6, lane 4). Analyses of cell lysates from AML and ALL patients revealed the presence of the NMU1R only in the AML sample (Figure 6, lanes 5 and 6). Taken together, these results suggest that NMU1R expression is specific to myeloid leukemia cells.

Intracellular Ca^{+2} Flux of NmU Treated K562 Cells. Biologically active NmU has been reported to bind specifically to NMU1R transiently expressed in CHO, HEK-293 and COS-7 cells and cause an increase in intracellular Ca^{+2} levels [42,47,48]. We therefore determined the ability of NmU to induce intracellular Ca^{+2} flux in K562 cells. A maximum Ca^{+2} flux of Indo-1 labeled K562 cells was observed at 167 $\pm$ 15 seconds after adding 300 μM of NmU (Figure 7), demonstrating that K562 cells express functional NMU1R. Sequential addition of first NmU and then thrombin, a known stimulator of

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intracellular Ca^{+2} flux in K562 cells [49], resulted in an intracellular Ca^{+2} flux following the addition of NmU but not with thrombin (data not shown), suggesting that NmU and thrombin induce Ca^{+2} flux in hematopoietic cells through the same pathway.

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DISCUSSION

c-Myb is a nuclear transcription factor required for hematopoiesis and has been postulated to play a role in leukemogenesis. However, the mechanism responsible for transforming hematopoietic cells remains unclear. We have previously reported that leukemic cells are more sensitive to antisense *c-myb* oligodeoxynucleotides than normal hematopoietic cells [35], suggesting that Myb-regulated genes might play a key role in maintaining the leukemic state. Similar results have been obtained using a monoclonal antibody to c-Myb [50]. To better understand Myb's role in leukemogenesis, we sought to identify Myb-regulated genes in human myeloid leukemia cells that were engineered to express a dominant negative Myb protein, MERT. Using a microarray strategy, we identified a potentially novel Myb gene target, NmU by virtue of the degree of change in its expression. Validation of this result was obtained by quantitative real-time PCR studies, which revealed a dramatic increase in the level of NmU expressed in primary blood cells from AML patients (Figure 3E).

Biologically active NmU is a 25-amino acid peptide that is expressed in such tissues as intestine, brain, and bone marrow [42]. It has a myriad of activities including the ability to stimulate smooth muscle contraction [51], local blood flow and ion transport in the intestine [52-54], adrenocortical function [55], and blood pressure [41]. NmU is also involved in the central control of appetite [56]. Our findings suggesting that NmU is a Myb regulated gene in myeloid leukemia cells prompted us to investigate the biologic significance of this expression.

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Since *c-myb* has significant effects on cell proliferation, we first examined the effect of NmU on cell growth. We found that the addition of NmU to K562 cells expressing MERT stimulated the growth of cell clusters that contained ~3-fold more cells than those cultured in the absence of NmU (Figure 4 and Table 2). Unfortunately, we were unsuccessful in culturing leukemic CFU. However, when NmU was added to suspension cultures of primary AML cells, cell proliferation was increased compared to growth of cells in the absence of NmU, in three out of four patient samples evaluated (Table 3). Further, when endogenous NmU expression was inhibited in myeloid leukemia cells by NmU siRNA, there was a statistically significant ($p<0.006$) ~2-fold decrease in cell viability 24-hours post-treatment compared to untreated cells (Figure 5). Taken together, these data support the hypothesis that NmU stimulates myeloid leukemia cell proliferation and suggest that NmU, and its cognate receptor, NMU1R, may be added to the list of autocrine growth loops that have been defined in leukemia cells, including the single-chain polypeptide type-1 insulin-like growth factor (IGF-1) and its receptor [57].

The precise mechanism whereby NmU stimulates cell growth is not entirely clear but is likely accomplished by rescuing cells from the block in cell cycle progression caused by Myb inactivation. This might ultimately be affected through events triggered by the intracellular Ca^{+2} flux we measured when cells were exposed to NmU. We note that when CHO, HEK-293 and COS-7 cells transiently expressing NMU1R are treated with NmU, an increase in intracellular Ca^{+2} flux is observed, as well as an increase in total inositol phosphate levels [42,47,48], indicating the NmU induces intracellular events via G-

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protein coupled receptor signaling. Signal transduction through G-protein coupled receptors has been reported to activate a number of intracellular pathways such as the RAS/MAPK pathway that leads to proliferation and the activation of PI-3 kinase [58] that leads to cell migration [59].

Finally, the chain of events that link Myb and NmU expression remains to be elucidated. We have noted that there are three potential Myb binding sites upstream of the putative transcriptional start site of human NmU suggesting that Myb could directly regulate NmU gene expression but this is not proven, and other intermediate steps are certainly possible. Studies to define the NmU promoter and investigate the direct or indirect interaction of Myb with this promoter are currently underway. In addition, while a certain threshold of Myb expression might be required for induction of NmU, other factors, likely tissue specific, could certainly play a role. For example, c-Myc is another Myb regulated gene whose expression is complex. Murine myeloid leukemia cells manifest a decrease in c-myc expression when they express a dominant negative Myb, along with a cell cycle arrest and an increase in apoptosis[10]. However, in a murine erythroleukemia cell line, a dominant negative Myb blocked differentiation, but had no effect on proliferation or c-myc expression [38]. In T cells, a dominant negative Myb augmented apoptosis but had no effect on c-myc expression [20].

In summary then, using K562 cells transduced with an inducible dominant negative Myb, we have been able to identify a previously unknown, Myb regulated autocrine growth loop of potential physiologic significance in myeloid leukemia cells. The loop consists of

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a small peptide, NmU, and its cognate receptor NMU1R. Experiments were conducted which suggest that this loop is functional in myeloid leukemia cells but as noted above, a number of questions concerning the mechanism of NmU regulation and its mechanism of action in hematopoietic cells remain to be determined. Answering these questions will help elucidate the function of NmU in hematopoiesis, and will help determine the ultimate value of NmU as a therapeutic target in acute leukemia.

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Table 1. Candidate Myb-gene targets

Accession # *	Genes suppressed by Myb†	Fold change
NM_000519	Hemoglobin, delta	4.3
M12530	Transferrin	3.2
NM_001706	B-cell CLL/lymphoma 6	3.1
U72649	B-cell translocation gene 2	3.0
M35999	Platelet glycoprotein IIIa	2.6
	Genes upregulated by Myb‡	
NM_006681	Neuromedin U	4.9
AI816485	Schneider fetal brain 00004 Homo sapiens cDNA clone	4.3
AF015592	Cdc7	2.8
NM_002156	Heat shock 60-kDa protein 1 (chaperonin)	2.8
AI928823	Histone H3	2.6

Ten of the 105 genes that changed in expression > 2-fold in tamoxifen treated K562-MERT cells compared to untreated cells are provided. The complete microarray data set was deposited in ArrayExpress (**www.ebi.ac.uk/arrayexpress/**). The accession number for the data set is E-MEXP-91.

* Accession # is the GenBank Accession number corresponding to each gene.

† The five genes listed to be suppressed by Myb are among the genes that increased the most in expression in tamoxifen treated K562-MERT cells as determined by microarray analysis.

‡ The five genes listed to be upregulated by Myb are among the genes that decreased in expression to the greatest extent in tamoxifen treated K562-MERT cells following microarray analysis.

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Table 2. Quantitation of K562-MERT colonies and cells in methylcellulose cultures

Treatment*	Colony number†	Cell number‡
None	840 ± 198	$1.70 \pm 0.223 \times 10^5$
NmU	780 ± 201	$5.21 \pm 0.303 \times 10^5$
NmU + 4-OHT	117 ± 61	$1.06 \pm 0.597 \times 10^5$
4-OHT	63 ± 24	$0.443 \pm 0.138 \times 10^5$

* K562-MERT cells were combined with a cytokine cocktail and methylcellulose as described in the "Materials and Methods". In addition, the methylcellulose cultures received either (1) "none" which is no additional reagents (2) "NmU" which is the addition of 35 μM NmU peptide, (3) "NmU + 4-OHT" which is the addition of both 35 μM NmU peptide and 1 μM 4-OHT, or (4) "4-OHT" which is the addition of 1 μM 4-OHT.

† K562-MERT colonies were scored on day 14. The number of K562-MERT colonies in each experimental condition is the mean of 8 individual determinations ± standard deviation.

‡ The total K562-MERT colonies per treatment were dissociated into individual cells with warm phosphate buffered saline and counted in a hemacytometer. The number of K562-MERT cells in each experimental condition is the mean of 4 individual determinations.

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Table 3. Effect of NmU on the proliferation of primary AML cells

Patient #[*]	Number of cells/ml[†] (-) NmU	Number of cells/ml[†] (+) NmU	*p* Value
UPN198	$5.42 \pm 1.10 \times 10^5$	$9.97 \pm 1.03 \times 10^5$	0.026
UPN228	$35.3 \pm 2.47 \times 10^5$	$45.5 \pm 0.710 \times 10^5$	0.002
UPN304	$0.880 \pm 0.113 \times 10^5$	$0.940 \pm 0.219 \times 10^5$	0.402
UPN321	$0.550 \pm 0.049 \times 10^5$	$0.830 + 0.028 \times 10^5$	0.010

[*] Patient # was assigned by the Stem Cell Core Facility at the University of Pennsylvania. UPN is unique patient number.

[†] Mean ± standard deviation of duplicate cultures. Measurements for UPN198, UPN228, UPN304, and UPN321 were taken on days 8, 6, 6, and 4 of culture, respectively, as described in the "Materials and Methods".

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FIGURE LEGENDS

Figure 1. K562-MERT colonies are smaller in the presence of tamoxifen. A mixture of K562 and K562-MERT cells were cultured in methylcellulose in the absence (A,B) and presence (C,D) of 1 μM of tamoxifen. After 14 days, the resulting colonies were examined by phase (A,C) and fluorescent (B,D) microscopy.

Figure 2. K562-MERT cells arrest in the G1/S phase of the cell cycle when Myb activity is inhibited. K562 (A,B) and K562-MERT (C,D) cells were cultured for 72 hrs in the absence and presence of 1 μM of tamoxifen. The DNA content in the PI stained cells was measured by flow cytometry and analyzed by ModFitLT Mac V3 software. The first shaded peak is the G0/G1-phase and the second shaded peak is the G2/M-phase of the cell cycle. The hatched area between the two peaks is the S-phase of the cell cycle. The insert contains the percentages of the total cell population in each phase of the cell cycle.

Figure 3. NmU expression is decreased in tamoxifen treated K562-MERT cells and is elevated in primary AML cells. Quantitative real-time PCR was used to quantitate the expression of NmU in K562-MERT (A) and K562 (B) cells and quantitate GAPDH expression in K562-MERT cells (C) that were untreated (⑥) and treated with tamoxifen (④) for 72 hrs. The levels of *c-myb* (*D*) and NmU (*E*) expressed in controls (n = 6) and patients with ALL (n = 7) and AML (n = 7) were also determined by quantitative real-time PCR. Total RNA was extracted from each sample, reverse transcribed, and

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amplified using gene specific primers and TaqMan probes as detailed in the "Materials and Methods." Real-time PCR was performed in triplicate for each cell line and subject, and the expression of each gene was normalized to 18S. The results are presented as the mean ratio of the number of NmU (A,B,E), GAPDH (C), and *c-myb* (D) copies to the number of 18S copies per μg of RNA.

Figure 4. NmU stimulates proliferation in K562-MERT cells. K562-MERT cells were cultured in methylcellulose with GM-CSF and IL-3 (A). In addition, some of the cultures received either 35 μM NmU (B), or 1 μM 4-OHT (C), or both 35 μM NmU and 1 μM 4-OHT (D). Colonies consisting of 20 or more cells were manually counted on day 14 using an Olympus IX 70 microscope at 10X magnification. Representative colonies from eight independent experiments are shown for each treatment at 10X magnification.

Figure 5. Inhibition of NmU gene expression inhibits proliferation of human myeloid leukemia cells. (A) Total RNA from K562 cells nucleofected in the absence (-) and presence (+) of siRNA was isolated 24 hours post-nucleofection, reverse transcribed, and amplified in triplicate using 18S (⑥) or NmU (④) primers and their corresponding TaqMan probes as described in the "Materials and Methods." (B) Percent cell viability of K562 cells that were mock nucleofected, or nucleofected with control siRNA (Dharmacon), or NmU siRNA was determined by trypan blue exclusion 24 hours post-nucleofection.

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Figure 6. The NmU receptor is expressed in human myeloid cells. The expression of the NMU1R was determined by Western blot analysis. Total protein (50 μg) from HL-60 cells (lane 1), peripheral blood mononuclear cells from a normal donor (lane 2), K562 cells (lane 3), K562-MERT cells (lane 4), pheresis from a representative AML patient (lane 5), and peripheral blood from a representative ALL patient (lane 6) were fractionated by 10% SDS-PAGE, transferred to a PVDF membrane and probed with anti-NMU1R. Along the left edge is the position of the protein standards and the arrow points to NMU1R. The solid white line between lanes 4 and 5 denotes the removal of irrelevant sample lanes from the same blot. The blot shown is representative of three individual determinations.

Figure 7. Biologically active NmU induces intracellular Ca^{+2} flux in K562 cells. The time course of the intracellular Ca^{+2} flux response to the NmU peptide was performed as described in the "Materials and Methods." The solid black arrows indicate the addition of NmU or ionomycin to Indo-1 labeled K562 cells. The results are representative of five independent experiments.

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FIGURE 1

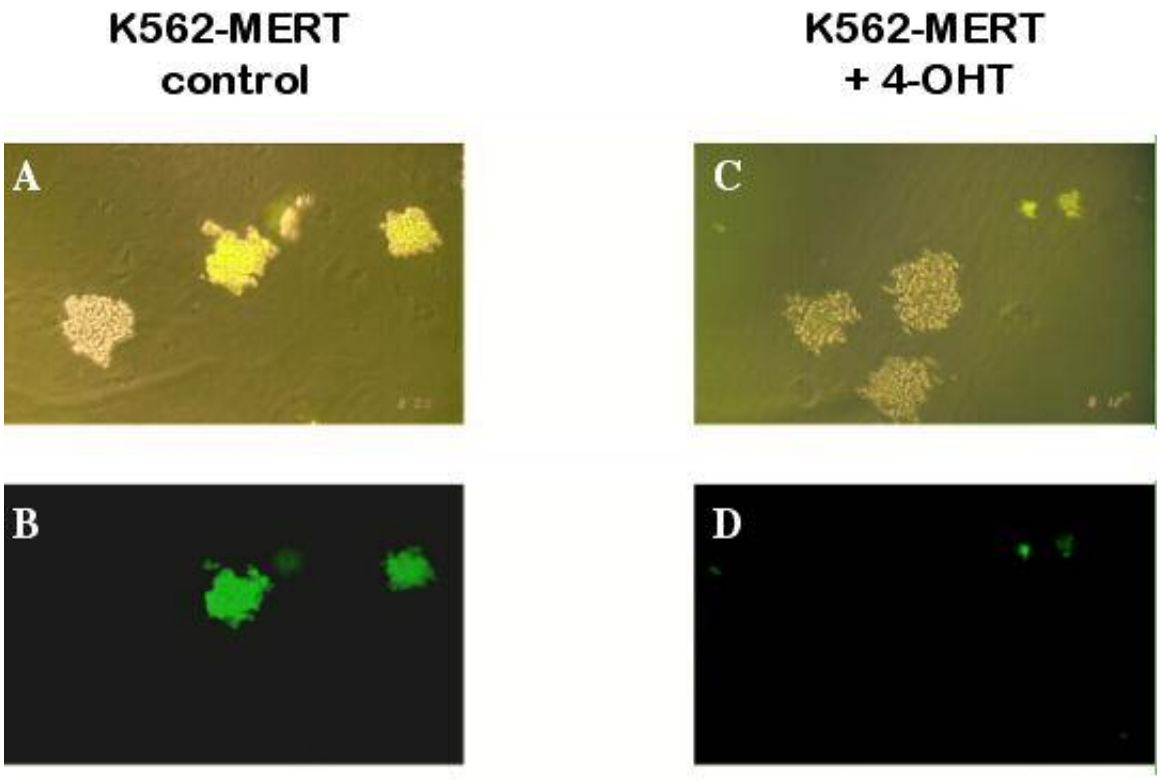

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FIGURE 2

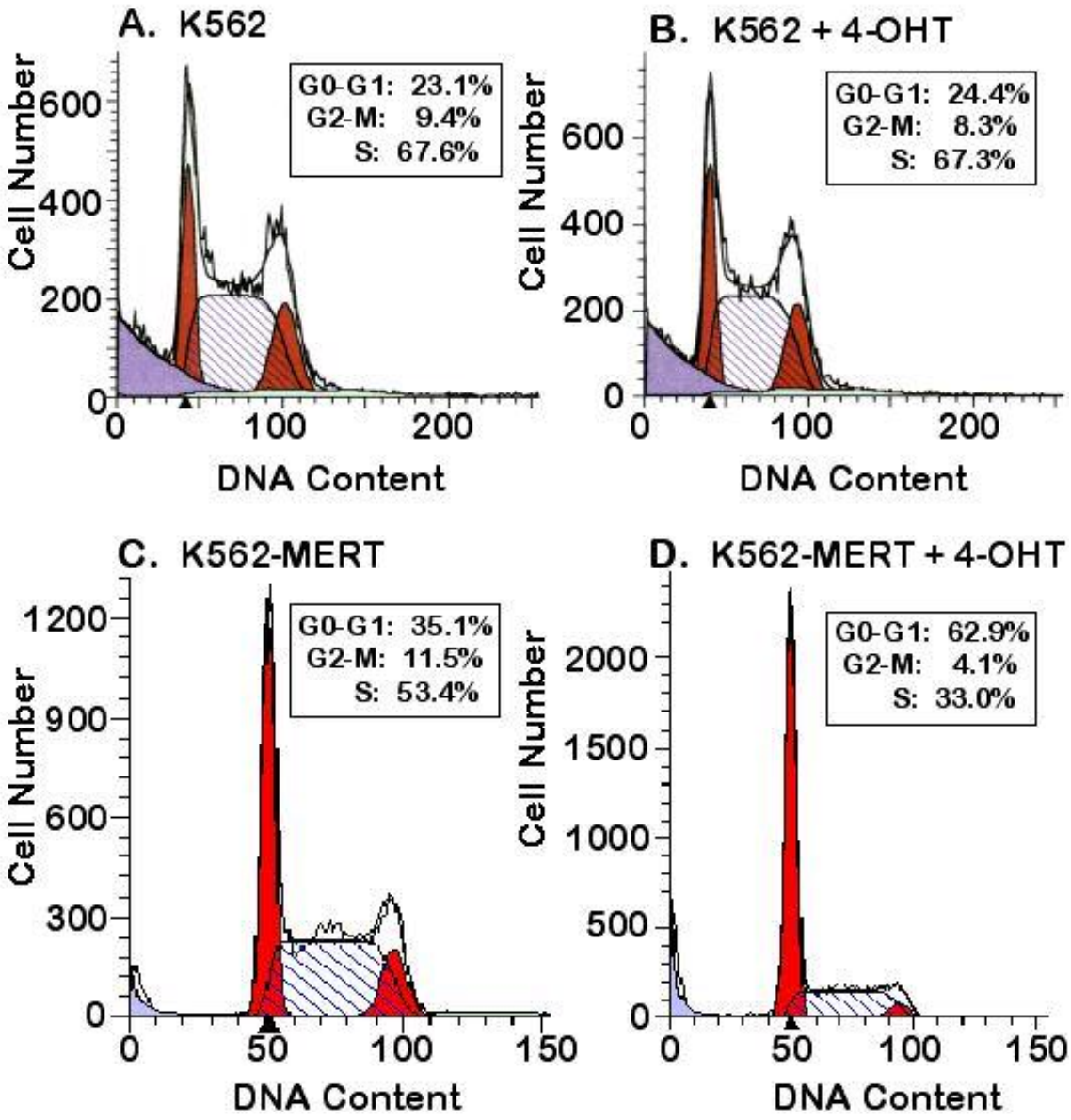

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FIGURE 3

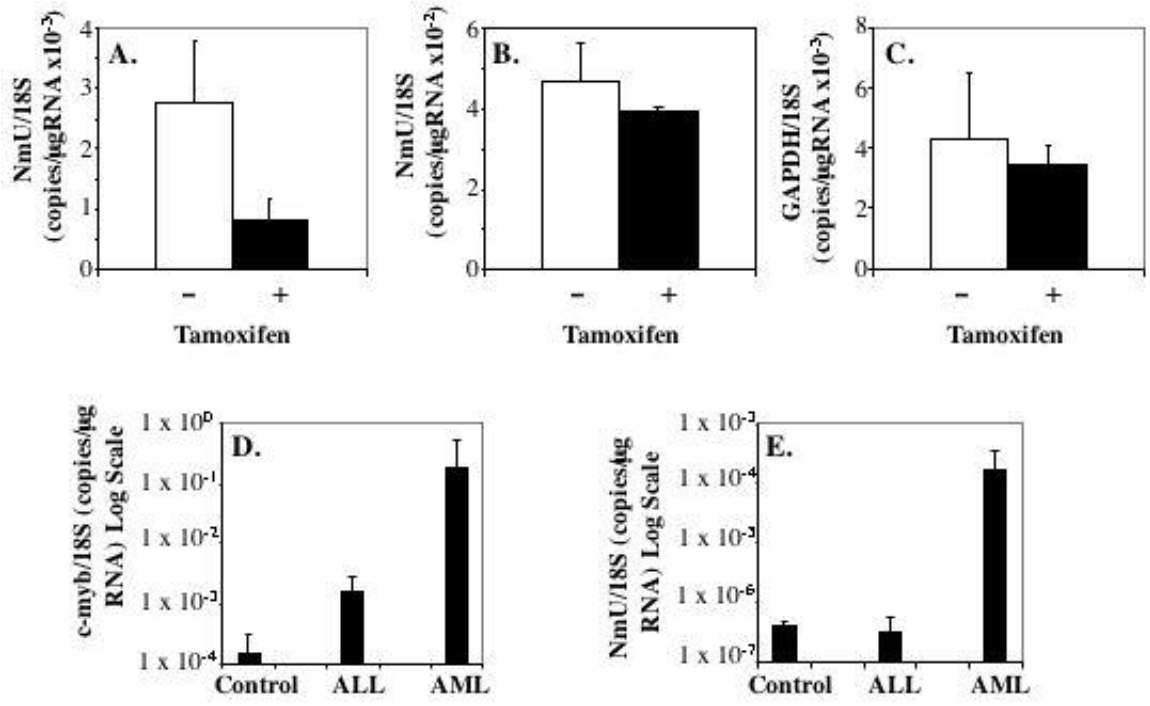

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FIGURE 4

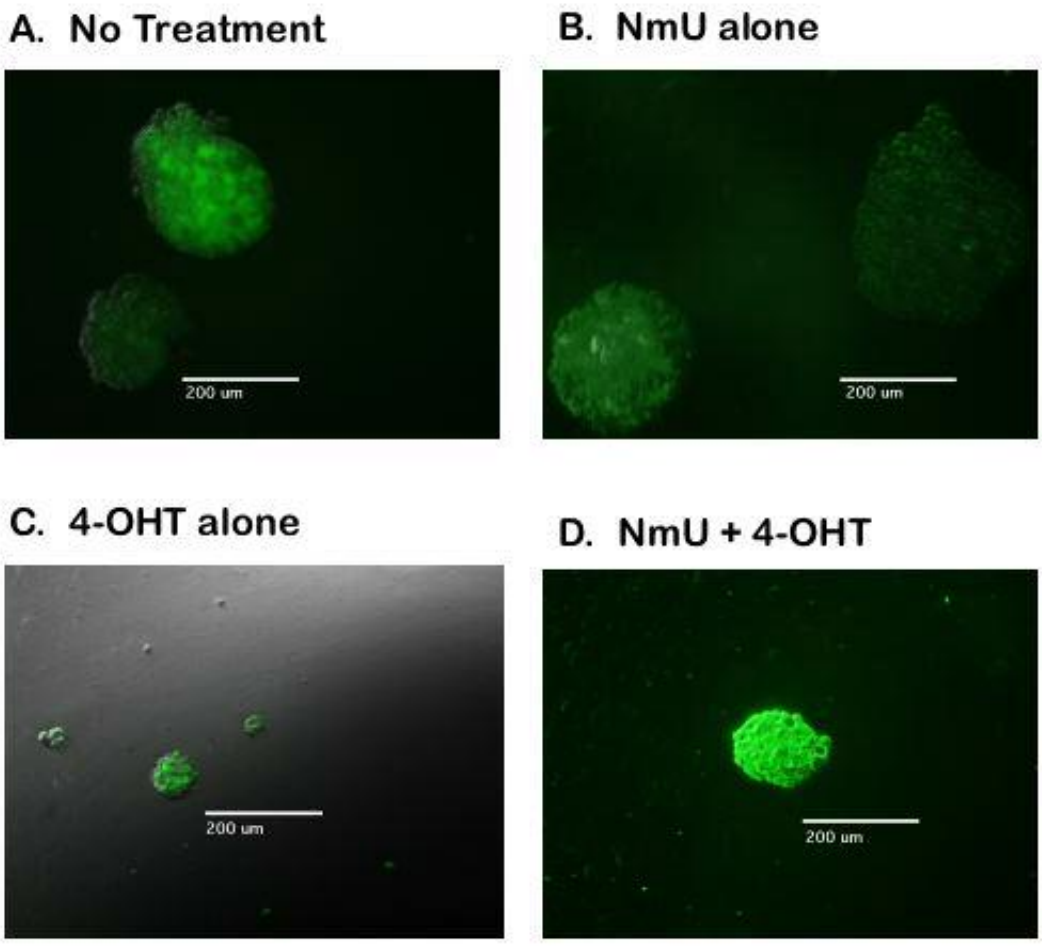

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FIGURE 5

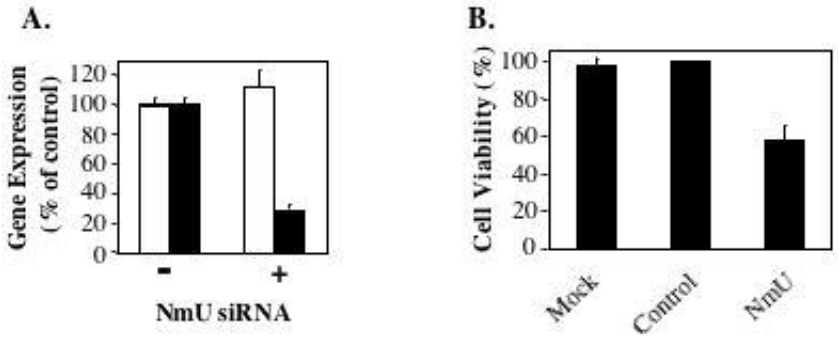

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FIGURE 6

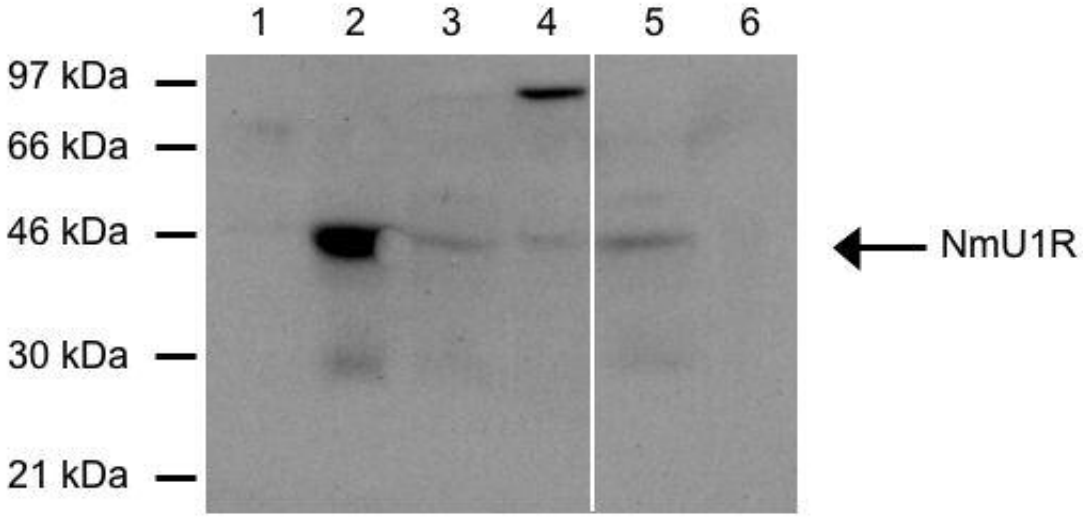

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FIGURE 7