Antitumor Activity of Sustained N-Myc Reduction in Rhabdomyosarcomas and Transcriptional Block by Antigene Therapy

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Abstract

Purpose: Rhabdomyosarcomas are a major cause of cancer death in children, described with MYCN amplification and, in the alveolar subtype, transcription driven by the PAX3-FOXO1 fusion protein. Our aim was to determine the prevalence of N-Myc protein expression and the potential therapeutic effects of reducing expression in rhabdomyosarcomas, including use of an antigene strategy that inhibits transcription.

Experimental Design: Protein expression was assessed by immunohistochemistry. MYCN expression was reduced in representative cell lines by RNA interference and an antigene peptide nucleic acid (PNA) oligonucleotide conjugated to a nuclear localization signal peptide. Associated gene expression changes, cell viability, and apoptosis were analyzed in vitro. As a paradigm for antigene therapy, the effects of systemic treatment of mice with rhabdomyosarcoma cell line xenografts were determined.

Results: High N-Myc levels were significantly associated with genomic amplification, presence of the PAX3/7-FOXO1 fusion genes, and proliferative capacity. Sustained reduction of N-Myc levels in all rhabdomyosarcoma cell lines that express the protein decreased cell proliferation and increased apoptosis. Positive feedback was shown to regulate PAX3-FOXO1 and N-Myc levels in the alveolar subtype that critically decrease PAX3-FOXO1 levels on reducing N-Myc. Pharmacologic systemic administration of the antigene PNA can eliminate alveolar rhabdomyosarcoma xenografts in mice, without relapse or toxicity.

Conclusion: N-Myc, with its restricted expression in non-fetal tissues, is a therapeutic target to treat rhabdomyosarcomas, and blocking gene transcription using antigene oligonucleotide strategies has therapeutic potential in the treatment of cancer and other diseases that has not been previously realized in vivo. Clin Cancer Res; 18(3); 796–807. ©2011 AACR.

Introduction

The Myc-family of transcription factors are involved in many critical cellular processes including cell proliferation and apoptosis and they are implicated in the development and progression of tumors (1). MYCN gene amplification is most widely known for its association with poor outcome in neuroblastomas (2), although other pediatric tumors including rhabdomyosarcomas (RMS) have been described with MYCN amplification (3–5). RMS are the most frequent soft tissue sarcomas in children with two main histologic subtypes, embryonal (ERMS) and alveolar (ARMS). The
Translational Relevance

Rhabdomyosarcomas are the most common pediatric soft tissue sarcoma. They are a leading cause of cancer death in children with few therapeutic options in high-risk categories. N-Myc is a transcription factor belonging to the MYC protein family that has a highly restricted expression pattern in non-fetal tissues. N-Myc protein expression in patient samples of rhabdomyosarcoma and an apoptotic response to sustained reduction of expression in rhabdomyosarcoma cell lines imply that N-Myc represents a tumor-specific therapeutic target in these tumors. Systemic treatment of mice with rhabdomyosarcoma cell line xenografts using an antigen peptide nucleic acid (PNA) oligonucleotide conjugated to a nuclear localization signal peptide that inhibits transcription of MYCN shows promise by eliminating tumors. This direct antigen approach may have therapeutic potential in the treatment of other cancers and diseases.

Materials and Methods

RMS tissue from patients and cell lines

Formalin-fixed, paraffin-embedded diagnostic tumor material from 79 patients with RMS was collected from U.K. centers through the Children’s Cancer and Leukemia Group (Local Research Ethics Committee protocol Nos. 1836 and 2015 and Multi-Regional Research Ethics Committee/06/4/71 with consent, where required). The histology was confirmed by review according to WHO guidelines to be 25 alveolar and 54 embryonal, and 0.6 mm diameter cores from 3 or more defined regions of tumor blocks were used to construct a tissue microarray (TMA). Patient data for this cohort are summarized in Supplementary Table S1.

Expression of MYCN is very restricted after birth (12), and the effects of reducing N-Myc levels in neuroblastoma cells (13, 14) have led to suggesting N-Myc as a therapeutic target for neuroblastomas. Various therapeutic strategies have been proposed, including indirect approaches that impact on the stability of the N-Myc protein (15, 16). However, therapeutic targeting of nuclear transcription factors such as those of the Myc-family is considered challenging. Oligonucleotides in clinical trials are based on antisense approaches which target mRNA and inhibit translation, whereas less well-known antigenic strategies target the chromosomal DNA and inhibit transcription (13, 17–22). Antigene approaches offer conceptual advantages over antisense: usually two gene copies per cell to target rather than multiple copies of mRNA that are continually being transcribed; a persistence block of the transcriptional process; and simultaneous inhibition of all splice variants and their simple and nonrestrictive design (13, 17–22). In keeping with requirements for clinical application, peptide nucleic acid (PNA) oligonucleotides (23) show potent antigenic activity (13, 17, 18, 21) and are resistant to nucleases and proteases although their in vivo potential has not been shown.

Here, we report the prevalence of N-Myc protein in a large series of RMS, dependence of RMS cell viability on N-Myc expression and, as a paradigm for in vivo antigenic therapy, the pharmacologic activity of an MYCN-specific antigen PNA to treat aggressive ARMS in mice.

MYCN gene copy number and N-Myc immunohistochemical analyses

FISH was conducted on the TMAs and nuclei from cell lines for fusion gene status and MYCN copy number as previously described (25) using probes for MYCN (2p24) and LAF (2q11; MP Biomedicals). The copy number of probes for MYCN (2p24) and LAF (2q11; MP Biomedicals) were scored in at least 20 nuclei from each core and amplification defined as greater than 4-fold excess of MYCN signals relative to LAF (5) with the maximum score from all cores from a patient or cell line used. Gain of MYCN was inferred when a 1- to 4-fold excess of MYCN or between 4 and 8 copies of both probes were scored. Immunohistochemistry on the TMAs used antigen retrieval with microwave treatment in target retrieval solution, pH 6.0 (Dako) for 20 minutes. Detection used the NCMI100 antibody against N-Myc (gift from Dr. Nao Ikegaki) at 1:10 dilution, Ki-67 (MIB1, 1:300 dilution; Dako), prediluted Universal Negative Controls (Dako), and the immPRESS Kit (Vector Laboratories) according to the manufacturers’ instructions. Cell lines known to express N-Myc protein were used as positive controls for the immunohistochemistry on the TMAs as well as primary material and cell lines that did not (normal skeletal muscle, normal liver), RMS cell line (MYCN amplified), and RD cell line (no N-Myc expression detected on Western blotting). Slides were only scored where convincing nuclear staining was obtained in the positive control cores and where negative controls remained negative. Positive and negative samples ensured successful staining, and each core was scored as 0 for no staining, 1 for weak, 2 for moderate, and 3 for strong staining in 10% or more of cells for categories 1 to 3. Each TMA was scored independently by two pathologists.

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(K. Thway and J.S. Reis-Filho). In rare cases of discrepancy, a consensus score was agreed after reanalysis.

**PNA design**

A selected optimal antigenic PNA (PNA-MYCN) complementary to unique sequence of the antisense strand of exon 2 was used (13). To test the specificity of PNA-MYCN, we also designed a mismatched PNA (PNA-MUT) containing a three-base substitution and an additional antigenic PNA (PNA-MYC) complementary to a unique sequence in the exon 2 of the antisense strand of MYC (16). PNAs were covalently linked to a nuclear localization signal (NLS) peptide (PKKKRKV) at their C-terminus. A fluorescently labeled PNA-MYC was also synthesized by linking a rhodamine (Rho) fluorophore to its N-terminus. Sequence information, synthesis, purification, and characterization of these PNAs are described in the Supplementary Methods. To evaluate the cellular uptake and intracellular localization of PNA-MYC, fluorescence microscopy analysis was conducted in RH30, RH4, and SMS cells using PNA-MYC conjugated with rhodamine (Rho-PNA-MYC; 10 μmol/L) as previously described (13).

**Electrophoretic mobility gel shift assay**

A complementary double-strand DNA (dsDNA) oligonucleotide (MYCNwt-36mer: 5’-CACTGTCCACCTGCGGCACTGATCGCCAGAACC-3’) was used, containing the target sequence for PNA-MYC and flanking sequences of the MYCN gene (GenBank accession no. M13241). DNA (4 μmol/L; each strand) and PNA-MYC (2 μmol/L) or PNA-MYC (NLS–) were incubated at 90°C for 10 minutes in PBS buffer (0.1 mol/L phosphate, 0.1 mol/L NaCl, 0.1 mmol/L EDTA) and then left to anneal at room temperature for 60 minutes. Gel electrophoresis used PAGE (acrylamide; BioRad Criterion) in TBE 1 mmol/L EDTA and then left to anneal at room temperature for 60 minutes. Gel electrophoresis used PAGE (15% bis-acrylamide; BioRad Criterion) in TBE 1×, voltage, 100 V with run time of 100 minutes and sample size of 25 mL. Staining was conducted with silver (0.2% silver nitrate in water) at room temperature for 90 minutes followed by development [0.3% (w/v) sodium carbonate; 0.025% (w/v) formaldehyde, 10 mg/L sodium thiosulfate], fixing (10% acetic, 40% ethanol) at room temperature overnight, and washing with water (doubly distilled). Images were acquired using BioRad GS800 Calibrated densitometer.

**PNA treatment, RNA interference, and cell line growth**

Experiments in triplicate used 96-well cluster plates, 5 × 10⁴ cells were plated with 100 μL of OPTI-MEM (GIBCO) containing 4% fetal calf serum, 2 mmol/L-glutamine, and 1% penicillin/streptomycin. Cells were first incubated for 12 hours to permit adherence to the wells. The PNA-MYC was added to cells at final concentrations of 1, 5, and 10 μmol/L, and cells were harvested and counted every 24 hours using the ATPlite Assay (PerkinElmer). To evaluate the specificity of PNA-MYC for MYCN, the cells were treated at 10 μmol/L (the selected optimal concentration) PNA-MYC, PNA-MUT, or PNA-MYC. A pool of 4 siRNAs targeting the MYCN mRNA (simYCN) was purchased from Dharmacon Research (sequences in Supplementary Methods). A total of 5 × 10⁵ cells per well were plated (96-well plate) with 100 μL of growth medium without antibiotics to have cells with 50% to 80% confluency at the time of transfection. The day after, for each transfection sample, simYCN (100, 250, 500 nmol/L, and 1 μmol/L) or scrambled was administered in 15 μL of OPTI-MEM without serum and mixed. A total of 0.5 μL Lipofectamine (Invitrogen) was then diluted in 15 μL of OPTI-MEM without serum and treatment was conducted by following the manufacturer’s instructions. Cells were then incubated at 37°C in CO₂ incubator for 6 hours before replacing with fresh complete medium. Cell counts and viability were determined every 24 hours, using the ATPlite assay.

In independent additional experiments using cell lines RMS, RH30, RH41, and RD, cells were plated at a density of 3 × 10⁴ in 24-well plates. These were transfected 24 hours after plating with 2 pools comprising 3 equally represented siRNAs (Dharmacon; Supplementary Methods) at a final concentration of 33 nmol/L using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions. Each individual siRNA was assessed for MYCN knockdown (data not shown) and combinations of siRNAs were further optimized. Nontargeting control pool #2 (Dharmacon) was used as a negative control. Cell quantitation assays following these siRNA transfections were conducted using CyQUANT NF assay (Invitrogen) in 96-well plates and analyzed on a VICTOR® 3 fluorometer plate reader (PerkinElmer) using the excitation/emission spectra at 485/535 nm.

Lentiviral particles for short hairpin RNA (shRNA) knockdown of MYCN expression were made by transfecting (using 60 mL Lipofectamine 2000) HEK293T cells in a T75 cm² flask with the lentiviral packaging vectors, pMD2.G (3 μg), pMDL/pRRE (5 μg), pRSV-Rev (2.5 μg Addgene), and either the MYCN shRNA vector (10 μg; TRCN0000020696, Sigma) or control shRNA vector (10 μg; SHC002, Sigma) in OPTI-MEM (Invitrogen) in a total volume of 10 mL. Media were replaced on day 1 and lentiviral containing media were collected on day 2, spun to remove cell debris, and the supernatant filtered through a 0.45-μm low protein binding filter (Millipore). The viral titer was calculated using the p24 ELISA (Cambridge Biosciences) and viral aliquots stored at −80°C. Optimal cell plating densities were determined as 0.25 × 10⁶ (RH30, RD), 0.5 × 10⁶ (RH41, CT10), 0.6 × 10⁶ (RMS-YM), or 1 × 10⁶ (RMS) per well in 6-well plates. After 24 hours of plating, cells were transduced at a multiplicity of infection of 5 to 10 in the presence of polybrene (4 μg/mL; Sigma) for 24 hours and after a further 24 hours, selected with 1 μg/mL puromycin for 3 days. Cell counts following shRNA transduction were conducted using a hemocytometer in the presence of Trypan Blue.

**Generation of cell line clones overexpressing N-Myc**

The MYCN transcript was HA tagged and cloned from RMS cells into the pcI-Neo vector (Promega) using the primers MYCN F: 5’-ACCATGTACCCATACGACGTCCAGACGTACCTAGTACCGCGAGCTGCTCCA-3’ and MYCN R: 5’-GGCAATGCATGGCAGAAG-3’ and fully sequenced.
The pCI-Neo-HA-MYCN construct was transfected into RH3, RH4, and RMZ-R2C cell lines using Lipofectamine 2000 (Invitrogen). Cells were trypsinized and placed into selective media containing geneticin at 400 μg/mL 48 hours posttransfection. Cells remained in selective medium for a further 9 days at which time all untransfected cells had died under selection. Cells were then lysed with Cell lysis buffer (Cell Signaling Technologies) and subjected to Western blotting.

**Real-time reverse transcriptase PCR, gene expression profiling, Western blot, cell-cycle, and apoptosis analyses**

Total RNA was extracted from cells using the RNeasy Mini Kit (QIAGEN), reverse transcribed, and gene expression levels quantified using SYBR Green or Vic and Fam labeling as detailed further in the Supplementary Methods. RNA was hybridized to Affymetrix HG-U133A 2.0 arrays according to the manufacturer’s protocols and analyzed as described in the Supplementary Methods. Western blot analysis for N-Myc, c-MYC, and MET was conducted in RH30 ARMS cells using total proteins or for PAX3-FOXO1, FOXO1, and p21 analyses using nuclear proteins as also detailed in the Supplementary Methods. Flow cytometric analysis of cell cycle was conducted as previously described (13) in RH30 cells (1 x 10⁶) at 24 hours after treatment with PNA-MYCN or PNA-MUT (10 μmol/L; 3 identical experiments). RH30 and RH4 cells (4 x 10⁵) were plated in slide flasks with 2 mL of OPTI-MEM (GIBCO) containing 4% fetal calf serum, 2 mmol/L L-glutamine, and 1% penicillin/streptomycin. PNA-MYCN or PNA-MUT was added at 10 or 20 μmol/L to the cells, and apoptosis analysis was conducted after 24 and 48 hours using the In Situ Cell Death Detection TUNEL Kit (Roche) according to the supplier’s instructions. Fluorescence microscopy analysis was conducted with a BX-51 microscope (Olympus; 3 identical experiments). Eight days after lentiviral transduction of the shRNA to reduce N-Myc in 3 ARMS and 3 ERMS cell lines, apoptosis assays were conducted using Caspase3/7 Glo (Promega) and read using an MLX luminometer and revelation MLX software (Dynex Technologies).

**In vivo antitumor activity studies**

Six-week-old CD1 nude mice were obtained from Charles River Laboratories. All the experiments were approved by the Scientific Ethical Committee of Bologna University (protocol nos. 47621-x/6 and 46903-x/6) and carried out in the CRBA (www.CRBA.it). A total of 7 x 10⁶ RH30 cells growing at logarithmic phase were centrifuged and resuspended in 100 μL of physiologic solution. Cell suspensions were injected subcutaneously in the dorsal tissue with an insulin syringe under anesthesia with Zoletil 50 (10 μL intramuscularly). This xenograft murine model of ARMS was monitored by \textit{in vivo} (FDG; microPET) scan 2 days after the inoculum of tumor cells, and according to our previous experience (26), those found negative were excluded from the protocol because of a low probability to engraft. Semi-quantitative analysis was conducted for each tumor identified using the target to background ratio (TBR) as previously described (26).

Mice were treated after 10 days from inoculation of the tumor cells, when the tumors reached the tumor mass of approximately 100 mm³ in size. Three treatment groups of mice were delineated: first group (8 mice) was treated with PNA-MYCN, second group (4 mice) with PNA-MUT, and third group (13 mice) was treated in the same way with placebo (physiologic solution). Mice were injected intraperitoneally at the concentration of 50 mg/kg of PNA-MYCN or PNA-MUT in 100 μL of physiologic solution alternatively in the right and the left side of abdomen. The administration schedule consisted of injections every 48 hours. The total treatment lasted 20 days. Food intake, body weight, and general behavior all were noted during the study.

**Toxicologic analysis**

For the toxicologic analysis of the PNA-MYCN treatment \textit{in vivo}, two mice 129 sv/J1 were obtained from our colony (Authorization from Ministry of Health Prot.1237/p) and maintained in the same conditions described for CD1 nude mice used for the antitumor analysis. The toxicologic analysis was made administrating PNA-MYCN on two immunocompetent mice (129 sv/J1) versus two mice treated with physiologic solution. PNA-MYCN was injected following the same schedule described above. During and before treatment, mice were kept in standard conditions, weight was monitored, and the site of infection was kept under observation for any skin irritation. Mice were sacrificed 24 hours after the last PNA-MYCN injection, and a complete necropsy was conducted with the histologic evaluation of the more relevant organs.

To evaluate the bone marrow cells, bone marrow touch was conducted while peripheral blood was collected. Both were stained with May–Grünewald–Giemsa staining. Free antigen PNA-MYCIN in serum was determined by high-performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) as detailed in the Supplementary Methods.

**Statistical analyses**

Correlations between copy number and immunohistochemical scores were assessed by \( \chi^2 \) test for trend analyses. Correlations with Ki-67 were assessed comparing no expression with any expression using the Fisher exact test and overall survival, where data were available, was investigated using Kaplan–Meier analyses. TBR at the first scan was compared with TBR at the second, third, and fourth scans (t test). Statistical significance between experimental values was determined by \( t \) test, and differences were considered significant if \( P < 0.05 \).
Results

N-Myc protein is frequently expressed in RMS and high levels are associated with amplification, fusion gene status, poor outcome, and proliferative capacity

We first determined the prevalence of N-Myc protein in ERMS and ARMS. Immunohistochemistry for N-Myc protein was positive in 55% of all RMS, 76% (41 of 54) of fusion gene–positive ARMS, and 45% (70 of 155) of ERMS (Fig. 1; Table 1; and Supplementary Table S2). FISH analysis determined the MYCN copy number in the primary samples (Fig. 1). A correlation between the MYCN copy number and immunohistochemical scores was significant in ARMS and ERMS samples (χ² test for trend, \( P = 0.039 \) and \( P < 0.0001 \), respectively), and in ARMS, the frequency of amplification was 25%, similar to that previously reported by us and others (3, 5). Fusion gene–positive ARMS were associated with higher N-Myc expression than ERMS (χ² test for trend, \( P < 0.00005 \)) and higher expression was associated with poorer clinical outcome in patients with ARMS (log-rank test, \( P = 0.012 \), \( n = 48 \)) by Kaplan–Meier analysis but was not significant within fusion positive (log-rank test, \( P = 0.077 \), \( n = 39 \)) or ERMS cases (log-rank test, \( P = 0.52 \), \( n = 149 \)). N-Myc expression was positively associated with the marker of proliferation, Ki-67, in both ARMS and ERMS (the Fisher exact test, \( P = 0.024 \) and \( P = 0.0028 \), respectively; Supplementary Table S3). Therefore, N-Myc protein is frequently expressed in RMS and high levels are associated with amplification, PAX3-FOXO1, poor outcome for samples with alveolar histology (irrespective of fusion gene status), and proliferative capacity.

To characterize cell lines for the functional studies, FISH analyses were also used to assess MYCN copy number (Fig. 1). The cell lines RH30, RMS, RH28 (with PAX3-FOXO1), and RC2 (with PAX7-FOXO1) had genomic amplification of MYCN, RH30 in a heterogeneous manner. The RH41 and RH4 lines (with PAX3-FOXO1) and the embryonal cell line CT10 had MYCN gain and the cell lines RD, RMS-YM, CCA, RH36, SMS, and RH3 had normal copies of MYCN.

Antigene PNA-MYCN causes potent and specific inhibition of MYCN transcription and translation

We then synthesized an optimal antigene PNA (agPNA) that specifically blocked MYCN transcription (13). MYCN has 3 exons (22), but because exon 1 has many transcriptional start sites (27), it was not appropriate to design agPNAs targeting these (20, 21). We selected an optimal sense agPNA (PNA-MYCN) targeting a unique sequence of
MYCN gene in exon 2. Because PNA-MYC is a sense sequence complementary to the MYCN antisense strand target sequence, its activity can only occur by inhibiting transcription. The anti-MYC agPNA was conjugated to an NLS peptide for delivery (13, 17, 18) that we show penetrates cells without requiring a transfection agent and localizes to the nucleus (Fig. 2A). Moreover, the electrophoretic mobility gel shift assay (EMSA) conducted on a 36mer dsDNA containing the target sequence showed that only the PNA conjugated to NLS is able to bind the target dsDNA (Fig. 2B), consistent with the critical role for the NLS peptide in dsDNA invasion (13, 17, 18, 28). PNA-MYC caused a potent block of MYCN transcription in RH30 alveolar cells that reduced protein levels (Fig. 2C and D).

**MYCN silencing by antigen PNA-MYC causes specific cell growth inhibition and apoptosis in RMS cells**

The effects of silencing MYCN using PNA-MYC were then assessed in cell lines representative of ARMS and ERMS. The specific block of MYCN transcription and reduction of

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<th>PAX3-FOXO1, %</th>
<th>PAX7-FOXO1</th>
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*Table 1. Frequency of MYCN copy number changes determined by FISH and N-Myc expression by immunohistochemistry of primary ARMS and ERMS patient samples in TMAs*

Figure 2. Anti-MYC antigen PNA-NLS causes potent and specific inhibition of MYCN transcription and translation. A, representative fluorescence microscopy analysis of PNA-MYC uptake by RMS cells (RH30, RH4, and SMS) treated for 2 hours with Rho-PNA-MYC. B, EMSA of dsDNA showing strand invasion by antigen PNA-NLS. Lanes from the left to the right: 50 bp marker; dsDNA (36mer containing the MYCN target sequence for PNA-MYC); ssDNA 36mer containing the target sequence plus PNA-MYC, without NLS; ssDNA 36mer containing the target sequence plus half equivalent of PNA-MYC, showing the mobility of the DNA/PNA-NLS duplex; dsDNA plus PNA-MYC without NLS; and dsDNA plus PNA-MYC. C, expression of MYCN mRNA assessed by real-time RT-PCR in MYCN-amplified ARMS cells (RH30) after treatment with PNA-MYC or PNA-MUT (mean of 3 separate experiments; bars, SD). D, representative Western blot analyses of experiments showing N-Myc expression in MYCN-amplified ARMS cells (RH30) after treatment with PNA-MYC or PNA-MUT. Ctrl, control.
N-Myc protein by PNA-MYCN (Fig. 2C) was followed by cell growth inhibition. The level of cell growth inhibition (Fig. 3B) positively correlated with basal MYCN expression levels (Fig. 3A; Pearson’s correlation coefficient, \( r^2 = 0.88, P = 0.0005 \)). \( G_1 \) cell-cycle arrest and apoptosis were also shown in ARMS cells (Fig. 3C and D). The lack of effect of PNA-MUT (a control PNA containing 3 point mutations in the PNA-MYCN sequence) on MYCN levels (Fig. 2C) and on the phenotype of cell lines (Fig. 3B) as well as no effect of PNA-MYCN in RD (Fig. 3B), which has no MYCN expression, is consistent with specificity. PNA-MYCN without the NLS peptide [PNA(NLS/C3/C3/C3)] did not inhibit MYCN.
in vitro validation of MYCN as a therapeutic target using RNA interference

To further show that loss of cell growth and viability was specific to N-Myc reduction, expression was reduced by RNA interference in multiple RMS cell lines with and without MYCN amplification. Cell numbers were reduced by transient reduction of MYCN using 3 independent pools of siRNAs (Supplementary Figs. S2 and S3) and knockdown using a lentiviral construct to deliver an shRNA. The sustained level of reduction by shRNA achieved (Fig. 4A and B) eliminated cells in all MYCN-expressing cell lines including ERMS cell lines (Fig. 4C). This resulted in 3- to 8-fold increases of caspase-3/7 activity in all cell lines tested except RD, which does not express MYCN (Fig. 4D). These results using shRNA knockdown were similar to the effects of PNA-MYCN although the time course for PNA-MYCN was shorter than those for shRNA MYCN reduction (Fig. 3B). In contrast, the siRNA approach inhibited cell growth but...

transcription (Supplementary Fig. S1A) or cell growth (data not shown), showing that the NLS is essential for the antigenic activity.

**In vitro validation of MYCN as a therapeutic target using RNA interference**

To further show that loss of cell growth and viability was specific to N-Myc reduction, expression was reduced by RNA interference in multiple RMS cell lines with and without MYCN amplification. Cell numbers were reduced by transient reduction of MYCN using 3 independent pools of siRNAs (Supplementary Figs. S2 and S3) and knockdown using a lentiviral construct to deliver an shRNA. The sustained level of reduction by shRNA achieved (Fig. 4A and B) eliminated cells in all MYCN-expressing cell lines including ERMS cell lines (Fig. 4C). This resulted in 3- to 8-fold increases of caspase-3/7 activity in all cell lines tested except RD, which does not express MYCN (Fig. 4D). These results using shRNA knockdown were similar to the effects of PNA-MYCN although the time course for PNA-MYCN was shorter than those for shRNA MYCN reduction (Fig. 3B). In contrast, the siRNA approach inhibited cell growth but...
was unable to eliminate tumor cells, even with a daily treatment regimen (Supplementary Figs. S2 and S3).

**Gene expression changes associated with MYCN silencing and regulation of PAX3-FOXO1 expression by MYCN in ARMS cells**

Global gene expression profiles of ARMS cells (RH30) after 12 hours of PNA-MUT treatment compared with untreated cells showed strong similarities (Pearson, $R^2 = 0.920$) consistent with few off-target effects. PNA-MYCN (Fig. 5A and B), and the specific silencing of MYC did not alter expression of the homologous gene (Fig. 5A and B), and the specific silencing of MYC expression by an antigene PNA (PNA-MYC) in ARMS cells did not affect MYCN expression (Supplementary Fig. S4). This shows that the antigene PNA were specific for each MYC gene family member. However, PNA-MYCN treatment induced differential gene expression (Pearson, $R^2 = 0.751$ and 0.73 vs. PNA-MUT and untreated cells, respectively) that was shown in gene ontology analysis to be related to key features of RMS biology, including cell proliferation and myogenesis (Supplementary Tables S4 and S5). Validation of differentially expressed genes was conducted by quantitative reverse transcriptase PCR (RT-qPCR, Fig. 5A) and Western blotting (Fig. 5B). Surprisingly, reducing N-Myc levels affected genes previously associated with expression of PAX3-FOXO1 or known direct downstream targets of PAX3-FOXO1 (6, 7, 10, 29–31) such as MET, a direct target of PAX3-FOXO1 (31) and $p21^{CDKN1A/CIP1}$, that is linked to PAX3-FOXO1 oncogenicity (32) and G1 arrest (Figs. 3C and 5A and B). Consistent with this, N-Myc reduction decreased expression levels of the fusion genes (Fig. 5A and B), but not PAX3 or PAX7 (Fig. 5A). Conversely, overexpression of HA-N-Myc in appropriate cell line increased levels of PAX3-FOXO1 and PAX7-FOXO1 protein 11 days posttransfection (Fig. 5C). Taken together with evidence for PAX3-FOXO1 regulating the transcription of MYCN (7, 10, 29, 30), our data are consistent with positive feedback mechanisms contributing to levels of these proteins.

**Antigene PNA-MYCN causes specific eradication of ARMS cells in mice**

Finally, we evaluated the *in vivo* antitumor activity of PNA-MYCN in a xenograft murine model of ARMS by real-time molecular imaging using microPET, with FDG as a probe (26). Treatment of mice with PNA-MYCN led to tumor elimination in 75% of cases and strongly reduced the tumor signal in the other 25% (Fig. 6). The TBR was significantly greater than 1 ($P < 0.05$), both compared with the placebo. These *in vivo* results were obtained without the need for continuous treatment, but systemic administration (intraperitoneal, 50 mg/kg) every 2 days for 20 days. Subsequent monitoring for 30 days by microPET did not show any reappearance of the tumor in all 75% of cases where
treatment had led to elimination and showed stabilization of the minimal residual tumor mass in the remaining 25% of mice. MYCN is hardly expressed in non-fetal mouse and human tissues and can be considered tumor specific. Preliminary toxicologic analysis of the human PNA-MYC in immunocompetent mice after the same treatment schedule up to 12 hours after injection (Supplementary Fig. S6). Pharmacokinetic analysis showed persistence of the intact PNA-MYC (8 mice) or PNA-MUT (4 mice) or placebo (13 mice); the treatment (intraperitoneal, 50 mg/kg) was administered once every 2 days for 20 days. P value is compared with placebo.

Discussion

In this study, we have shown that N-Myc, with its restricted expression in non-fetal tissues, represents a potential tumor-specific therapeutic target in RMS. Despite this transcription factor generally being considered a challenging target, we provide evidence that antigen therapy targeting MYCN transcription has potential in treating tumors in vivo through systemic administration of MYCN-specific antigen PNA. We show that N-Myc protein is expressed in more than half of RMS samples in a large cohort. High levels significantly correlated with an increase in copy number of MYCN and/or the presence of the PAX3-FOXO1 or PAX7-FOXO1 fusion genes. The correlation of N-Myc expression with clinical outcome in cases with alveolar histology is consistent with fusion gene–positive cases, which express N-Myc highly, generally having a poorer outcome than fusion gene–negative alveolar cases (6, 7, 9). Seventy-seven percent of fusion-positive cases were immunohistochemically positive for N-Myc and all cases with the fusion gene that we have previously tested by quantitative RT-PCR show some level of MYCN expression (ref. 5; and data not shown). Therefore, a high proportion of RMS samples have the N-Myc target present.

Both N-Myc and the fusion proteins are known to play pivotal and co-operative roles in the biology of ARMS (7, 10, 11, 30, 33, 34). An association of N-Myc expression with fusion gene positivity is consistent with previous studies that have shown that MYCN is a direct downstream target of the PAX3-FOXO1 fusion protein (7, 10, 29, 30). However, further to this, we have shown that N-Myc modulation clearly alters expression of PAX3-FOXO1 and PAX7-FOXO1 at the RNA and protein levels and affects known downstream targets of PAX3-FOXO1. These include MET, a direct target of PAX3-FOXO1 (but not N-Myc), that decreases in expression levels after N-Myc reduction and is known to play an important role in RMS development in mice (35, 36) and is associated with tumor invasiveness (37, 38). N-Myc regulation of PAX3 promoter activity has been shown to involve a noncanonical E-box site in the 5' promoter region of PAX3 (39). However, we have shown that N-Myc reduction decreased fusion gene expression without changes to PAX3 levels. This implies that regulation of PAX3 and fusion gene expression levels by N-Myc is complex, and regulation of PAX3 by microRNAs has recently been described (40, 41).

Silencing MYCN expression induced and repressed a number of cancer-related genes linked to gene ontology features including myogenesis and cell proliferation, for example, PLK4 (Polo-like-kinase-4), a major regulator of centriole formation and associated with centrosome amplification (42, 43), decreased. This is consistent with the report that PLK4 and MYCN are overexpressed in PAX3-FOXO1–positive ARMS from transgenic mice (44). The p53 pathway is frequently inactivated in RMS through somatic mutations or functional inactivation (45). Remarkably, MYCN silencing restored p53-independent transcriptional regulation of major p53 downstream target genes in ARMS, both in terms of repression (such as PLK4 that is strongly
repressed by p53 in tumors; ref. 46) and activation (such as p21, BTG2, and GDF15). In all RMS cell lines, reduction of N-Myc caused upregulation of p21CDKN1A/CIP1, a known repressive transcriptional target of Myc (47). N-Myc promotes progression from G1 to S-phase of the cell cycle, whereas p21 promotes G1 arrest which is consistent with our observation that N-Myc reduction caused an accumulation of cells in the G1 phase.

The apoptotic responses observed in RMS cell lines after sustained inhibition of N-Myc are similar to that reported for neuroblastoma-derived cells (13, 14) as well as the apoptosis following inhibition of PAX3-FOXO1 expression in ARMS cell lines (48). In this study, we adopted an antigene strategy to persistently inhibit transcription of MYCN, that is induced by PAX3-FOXO1 protein in ARMS (10, 11). An antigen approach targets the gene in contrast to antisense that inhibits continually transcribed mRNA species. We have shown that the PNA-MYCN antigen fulfills many requirements of an effective drug candidate including specificity, cellular and nuclear uptake, with the NLS peptide also enhancing the stability of the PNA:DNA duplex (19), allowing the PNA-MYCN to strand-invade the target DNA sequence. Bioavailability at 12 hours after injection and lack of toxicity were also shown in mice in our investigations, although more extensive analyses will be required to comply with regulatory bodies. The minimum time to obtain a major MYCN mRNA decrease using the PNA-MYCN was shorter than shown by RNA interference approaches which may be explained by the fact that the transcriptional block by the antigen PNA peptide does not require the activation of protein complexes involved in mRNA target degradation used in antisense approaches, such as the RISC complex or RNase-H. Furthermore, silencing of MYCN by antigen PNA-MYCN led to complete tumor cell loss in vitro through PNA-MYCN administration only every 3 days, whereas treating with siMYCN required daily treatment that was unable to eliminate all tumor cells. Similarly, a noncontinuous administration (every 2 days) in vivo with antigen PNA-MYCN eliminated tumors in a previously validated xenograft model (26). The higher efficacy and persistence of the pharmacologic effect observed by the antigene approach suggests that from a therapeutic perspective, fewer doses than antisense would be required.

Complex algorithms based on mRNA structures are generally required to make effective antisense oligonucleotides. The optimal design of potent and specific antigen PNA oligonucleotides that will inhibit expression of mRNA splice variants appears relatively simple (13, 18, 21), for example, the design of an antigen PNA that effectively targets a transcriptional start site of the human progesterone receptor (21). The unique properties of the antigen approach are a potential alternative or complementary strategy to antisense and conventional pharmaceutical approaches. The dependence of RMS cells on MYCN expression shown here that was effectively targeted by PNA-MYCN both in vitro and in vivo shows promise for the treatment of clinically aggressive RMS and other tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

R. Tonelli and J. Shipley designed the study and jointly wrote the manuscript. R. Tonelli supervised experiments related to the PNA and some of those involving siRNAs. J. Shipley oversaw all other investigations.

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References

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