N-MYC Regulates Focal Adhesion Kinase Expression in Human Neuroblastoma*

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N-MYC is a transcription factor that plays an important role in cellular survival in neuroblastoma, and amplification of the N-MYC oncogene is the primary adverse prognostic indicator for neuroblastoma. Focal adhesion kinase (FAK) is a survival factor that has been shown to be overexpressed in many types of human cancers. In this study, we investigated the role of N-MYC regulation of FAK expression in neuroblastoma. We first found a correlation between N-MYC and FAK expression in neuroblastoma. Real time quantitative PCR demonstrated an increase in FAK mRNA abundance in the N-MYC-expressing neuroblastoma cell lines compared with the nonamplified SK-N-AS neuroblastoma cell lines. FAK protein expression also correlated positively with N-MYC expression in the N-MYC-amplified IMR-32 versus nonamplified SK-N-AS neuroblastoma cell lines. The same results were seen with the isogenic N-MYC+ (Tet−) and N-MYC− (Tet+) neuroblastoma cell lines. Promoter-reporter assays showed that activity of the FAK promoter was increased in the N-MYC-amplified IMR-32 cell line, in the N-MYC-transfected SK-N-AS nonamplified cell line, and in the isogenic N-MYC+ (Tet−) neuroblastoma cell lines compared with the nonamplified and N-MYC-nonexpressing cell lines. We also identified two N-MYC binding sites in the FAK promoter sequence and showed binding of N-MYC transcription factor to the FAK promoter through electrophoretic mobility shift, chromatin immunoprecipitation, and dual luciferase assays. Finally, down-regulation of FAK expression in N-MYC-inducible neuroblastoma cell lines with FAK small interfering RNA or a dominant-negative FAK protein (FAK-CD) significantly decreased viability and increased apoptosis in the N-MYC+ (Tet−) cells compared with the isogenic N-MYC− (Tet+) cells, demonstrating the biological significance of FAK overexpression in the N-MYC-expressing cell lines. This is the first report linking N-MYC and FAK in neuroblastoma, and it clearly demonstrates that N-MYC induces FAK expression. The results indicate that N-MYC regulation of FAK expression can control cellular functions in isogenic N-MYC−/+ (Tet+/−) neuroblastoma cell lines.

N-MYC is a transcription factor important for the control of cellular differentiation and proliferation (1, 2). It is normally expressed in the brain and peripheral nervous system (1). N-MYC has been shown to influence the differentiation of neural crest cells. Early in mouse embryogenesis, N-MYC is present primarily in the migrating neural crest cells, but as the embryo matures, the expression of N-MYC becomes limited to those cells that are undergoing neuronal differentiation (3). Abnormal expression of N-MYC is most notably associated with the pediatric tumor of neural crest origin, neuroblastoma. Amplification of the N-MYC oncogene is the primary adverse prognostic indicator in human neuroblastoma (4, 5). The level of N-MYC expression has been shown to correlate with the growth (6–9) and invasiveness (10) of neuroblastoma cells, and transgenic mice with N-MYC overexpression develop spontaneous neuroblastoma tumors (11). In addition, down-regulation of N-MYC with antisense oligonucleotides leads to decreases in both cellular proliferation and in anchorage-independent growth in the neuroblastoma cells (6, 9). Despite this information, the exact function and transcriptional gene targets of N-MYC in neuroblastoma are currently not well characterized (5).

Focal adhesion kinase (FAK)3 is a nonreceptor cytoplasmic 125-kDa protein-tyrosine kinase. Initial studies revealed that both the transcription of FAK mRNA (12) and the expression of FAK protein (13–19) are significantly increased in primary and metastatic breast, colon, and thyroid tumors compared with normal tissues and that these changes occur early in tumorigenesis. Real time PCR analysis of colorectal carcinoma and liver metastasis with matched normal colonic tissues demonstrated increased FAK mRNA abundance in the tumors and metastatic tissues compared with control tissues (20), suggesting that the increased FAK expression in human tumors occurs at the level of transcription. Recently the FAK promoter was cloned and characterized, and transcriptional regulation of the FAK promoter has been demonstrated (21).

FAK controls a number of cell signaling pathways including proliferation, viability, motility, and survival (22–25). The inhibition of FAK with antisense oligonucleotides has been shown to cause decreased growth in tumor cells (26). In addition, FAK inhibition with a dominant-negative FAK protein (FAK-CD) inhibited cell growth in human melanoma cells (12) and in

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3 The abbreviations used are: FAK, focal adhesion kinase; ChIP, chromatin immunoprecipitation; siRNA, small interfering RNA; EMSA, electrophoretic mobility shift assay; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Tet, tetracycline.
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human breast cancer cell lines (13, 27). Silencing FAK expression with small interfering RNAs resulted in decreased migration of lung cancer cells (28) and glioblastoma cells (29). Finally FAK silencing has been shown to lead to apoptosis in human fibroblasts (30) and ovarian cancer cells (31).

In the current study, we found significant expression of FAK mRNA and protein in neuroblastoma cell lines with N-MYC amplification and overexpression. These findings prompted us to investigate the binding of N-MYC to the FAK promoter and N-MYC regulation of FAK expression. Using the TRANSFAC (National Institutes of Health) (helixweb.nih.gov/transfac/index.html) and MatInspector (Genomatix Software GmbH) software, we analyzed the FAK promoter for the presence of N-MYC binding sites and found two potential sites: site 1 (−67 to −62) and site 2 (−181 to −176) in the P-280 region. By dual luciferase assays we showed that N-MYC-expressing and N-MYC-amplified neuroblastoma cells have increased FAK promoter activity compared with N-MYC-nonexpressing and N-MYC-nonamplified cell lines. We demonstrated that N-MYC binds to these sites both in vitro and in vivo by electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation (ChIP), respectively. Cellular viability studies showed that N-MYC+ (Tet−) cells have increased viability compared with their isogenic N-MYC− (Tet+) counterparts. In addition, inhibition of FAK expression in the N-MYC+/− (Tet−/+ ) isogenic cell lines with FAK siRNA or FAK dominant-negative AdFAK-CD (12, 13, 27) resulted in a significant decrease in viability and a significant increase in apoptosis in the N-MYC+ (Tet−) cells compared with their N-MYC− (Tet+) isogenic counterparts. This is the first study to link N-MYC with FAK in neuroblastoma, and it clearly demonstrates that N-MYC increases FAK expression. The findings in these studies also show a biological advantage to the N-MYC regulation of FAK expression.

**EXPERIMENTAL PROCEDURES**

**Cells, Cell Culture, and Transfections**—SK-N-AS, SK-N-DZ, and IMR-32 (32–34) neuroblastoma cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 1 μg/ml penicillin, and 1 μg/ml streptomycin. SK-N-BE(2) (33, 34) neuroblastoma cells were maintained in a 1:1 mixture of minimum Eagle’s medium and Ham’s F-12 medium with 10% fetal bovine serum, 1 μg/ml penicillin, and 1 μg/ml streptomycin. The Tet-off N-MYC+/− cell line (Tet-21/N or SHEP-21/N) was generously provided by Dr. S. L. Cohn (Northwestern University’s Feinberg School of Medicine, Chicago, IL) with permission from Dr. M. Schwab (Deutsches Krebsforschungszentrum, Heidelberg, Germany) (35). These cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1 μg/ml penicillin, and 1 μg/ml streptomycin and grown in the presence or absence of tetracycline (1 μg/ml) for 48–72 h for N-MYC− (Tet+) and N-MYC+ (Tet−) cells, respectively. Transfections of plasmids were done with Lipofectamine (Invitrogen) following the manufacturer’s instructions. Equal amounts of DNA were used for each transfection.

**DNA Constructs**—A plasmid encoding the N-MYC protein in a mammalian expression vector was produced by directional cloning of blunt end PCR products into a pcDNA 3.1D/V5-HisTOPO vector (Invitrogen). We used primers for cloning full-length N-MYC (5′-142CACCATGCGCGACGTCCAGCCTGCCAG-3′) and 3′-end primer for the coexpression of V5 and His6 tags (5′-GCAAGTCGGCTGTGTCAATTTTCC). All plasmids were sequenced at the Automated DNA Sequencing Facility at the University of Florida, and the expression of selected plasmids was analyzed after transfection into 293 cells with tag-specific and N-MYC-specific antibodies. FAK luciferase promoter constructs P-Basic, P-50, P-109, P-280, P-564, P-723, P-1020, and P-1173 were described in detail previously (21) and used for dual luciferase promoter-reporter assays as described previously (21).

**Antibodies and Reagents**—Monoclonal anti-FAK (4.47) and clone NCM II 100 anti-N-MYC antibodies were obtained from Upstate Biotechnology and Calbiochem, respectively. Myc tag antibody (polyclonal, ChIP grade) was obtained from Abcam, Inc. Monoclonal anti-β-actin antibody was obtained from Santa Cruz Biotechnology. SK-N-BE(2) and Kelly neuroblastoma cell nuclear extracts were obtained from Active Motif, Inc.

**Western Blotting**—Western blots were performed as described previously (36). Briefly antibodies to FAK (4.47, Upstate Biotechnology), N-MYC (Calbiochem), and β-actin (Santa Cruz Biotechnology) were used according to the manufacturers’ recommended conditions. Molecular weight markers were used to confirm the expected size of the target proteins. Immunoblots were developed with chemiluminescence Renaissance reagent (PerkinElmer Life Sciences). Blots were stripped with stripping solution (Bio-Rad) at 37 °C for 15 min and then reprobed with selected antibodies. Immunoblotting with antibody to β-actin provided an internal control for equal protein loading.

**Real Time RT-PCR**—Total cellular RNA was extracted utilizing the RNeasy kit (Qiagen) according to the manufacturer’s instructions. For the first strand synthesis of cDNA, 5 μg of RNA was used in a 20-μl reaction mixture utilizing a cDNA Cycle kit (Invitrogen) according to the supplier’s instructions. Resulting reverse transcription products were diluted 10 times and stored at −20 °C until later use. For TaqMan quantitative PCR, the following protocol was used. TaqMan PCR primers and probes for FAK and the housekeeping gene cyclophilin A were obtained from Applied Biosystems. Probes are labeled with a reporter dye, 6-carboxyfluorescein phosphoramidate at the 5′-end and with 6-carboxytetramethylrhodamine as a quencher dye at the 3′-end. TaqMan PCR was performed with 10 ng of cDNA in a 50-μl reaction volume containing TaqMan Universal PCR Master Mix using TaqMan gene expression assay (Applied Biosystems). Amplification was performed utilizing an ABI PRISM 7700 sequence detection system (Applied Biosystems). Cycling conditions were 50 °C for 2 min, 95 °C for 10 min followed by a 40-cycle amplification at 95 °C for 15 s, and 60 °C for 1 min. The ABI PRISM 7700 cycler software calculates a threshold cycle number (Ct) at which each PCR amplification reaches a significant threshold level. The threshold cycle number is proportional to the number of FAK RNA copies present in the reaction mixture. Experiments were repeated at least three times, and samples were analyzed in triplicate with
cyclophilin A utilized as an internal control. Data were calculated utilizing the ΔΔCt method (37) and are reported as mean -fold change ± S.E.

**Luciferase Promoter-Reporter Constructs and Dual Luciferase Assay**—Cells were plated in 6-well culture plates to ~80% confluence 24 h prior to transfection. Transfection was performed with pGL3 plasmids (1 μg/well) using Lipofectamine (Invitrogen) transfection agent according to the manufacturer’s protocol. A pRL-TK control vector containing the herpes simplex virus thymidine kinase promoter encoding Renilla luciferase resulting in its constitutive expression in the cells (21) (Promega) was used for normalization of luciferase activity. The pRL-TK vector was used (0.1 μg/well) together with the pGL3 plasmids for co-transfection. The level of firefly luciferase activity was normalized to that of the Renilla luciferase activity in each experiment. For all experiments, cells were cultured for 48 h after transfection and lysed with 1× passive lysis buffer (Promega). Lysates were analyzed using the dual luciferase reporter assay system kit (Promega). Luminences was measured on a TD20/20 luminometer (Turner Designs), and all experiments were performed at least in triplicate. Data are reported as mean -fold change in luciferase activity ± S.E.

**Site-directed Mutagenesis**—Site-directed mutation of the potential N-MYC binding site on the FAK promoter was obtained with a QuickChange SL site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol. The oligonucleotides for the mutant N-MYC site (with 6 bases of the site deleted (underlined)) were the following: forward, 5′-GAGCTTAGCGCCGCGCTGGG(A6)CGGGGGCGGCGCGCATGCCC-3′; and reverse, 5′-GGGCTAGCGCCGCGCGCGCCCCTGCC(A6)CCCCAGCGCGCGCTAGGCC-3′. These were then utilized to construct pGL3 mutant plasmids as described previously (21). All mutant plasmids were sequenced at the Automated DNA Sequencing Facility at the University of Florida.

**Chromatin Immunoprecipitation**—Cells were cultured to 80% confluence and then rinsed with phosphate-buffered saline and cross-linked for 15 min at 37 °C in serum-free medium containing 1% formaldehyde. Cultures were washed, and cells were collected by scraping into phosphate-buffered saline containing protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride and 1 μg/ml each of aprotinin, pepstatin B, and leupeptin). Cell pellets were resuspended in 1 ml of lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.0) containing protease inhibitors and incubated on ice for 10 min. DNA was then sheared by sonication to an average size of 300–1000 bp, and samples were centrifuged. Supernatants (0.2 ml) were diluted 1:10 with ChIP Dilution Buffer (Upstate) and 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, and 16.7 mM Tris containing protease inhibitors, and 5% was set aside as input DNA. Samples were then precleared for immunoprecipitation by incubation with salmon sperm DNA/Protein A-agarose slurry (Upstate) after 1 h of rotation at 4 °C. The collected Protein A-agarose-antibody-chromatin complex was washed three times for 5 min with Low Salt Immune Complex Wash Buffer (Chromatin Immunoprecipitation ChIP Assay Kit, Upstate), High Salt Immune Complex Wash Buffer (Chromatin Immunoprecipitation ChIP Assay Kit, Upstate), and Immune Complex Wash Buffer (Chromatin Immunoprecipitation ChIP Assay Kit, Upstate) and two times with Tris-EDTA buffer. The immune complexes were eluted from the antibody by incubating with 1% SDS and 0.1 mM NaHCO3 for 15 min at room temperature two times. To reverse the protein-DNA cross-links the combined eluates were heated with 0.2 M NaCl for 4 h. Samples of input starting DNA were made before the immunoprecipitation step by collecting the sonicated cell supernatant fraction in ChIP Dilution Buffer and reversing protein-DNA cross-links by adding 0.3 M NaCl and incubating for 4 h at 65 °C. DNA was purified using phenol-chloroform extraction and ethanol extraction and used for PCR analysis. PCR analysis was performed to identify the binding of N-MYC to the FAK promoter with the following primers: forward, 5′-TCACTTCTGTCTAAAGCCC-3′; and reverse, 5′-GGGACTTAGAAGTCCTGAG-3′. As a control, ChIP assay was also performed with the published telomerase promoter sequence (hTERT), and PCR was performed with the following primers: forward, 5′-AGTTGGATTGCAGCCGACAGA-3′; and reverse, 5′-TTCACCTGGCAGCAGA-3′ (38).

**Electrophoretic Mobility Shift Assay**—Nuclear extracts of SK-N-AS and N-MYC(−) Tet(+) cells were prepared with nuclear and cytoplasmic extraction reagents (NE-PER) (Pierce) according to the manufacturer’s protocol. Nuclear extracts from SK-N-AS(2) and Kelly neuroblastoma cells (amplified copies of N-MYC) were obtained from Active Motif, Inc.

The oligonucleotides were used as the consensus E-box sequence with the sequence shown underlined (5′-TGGCCGCACGCAGCGGGC-3′) (39), the focal adhesion kinase E-box N-MYC 1 sequence with the sequence shown underlined (5′-TGCCGCGCACGCGGGCGGG-3′), and the focal adhesion kinase E-box N-MYC 2 sequence with the sequence shown underlined (5′-CGCTGGGCATGGCCGGG-3′). Oligonucleotides were hybridized with complementary synthesized oligonucleotides and used in double-stranded form for labeling reactions. Oligonucleotides were labeled utilizing the biotin 3′-end DNA labeling kit from Pierce according to the manufacturer’s instructions. The DNA binding reaction of biotin-labeled, double-stranded oligonucleotides with 3 μg of nuclear protein extracts was done at room temperature for 20 min according to the manufacturer’s protocol. DNA-protein complexes were visualized with Novex 6% DNA retardation gels (Invitrogen). Electrophoretic mobility shift assays were performed with the LightShift chemiluminescence kit from Pierce according to manufacturer’s recommendations. Biotin-labeled DNA was then detected by chemiluminescence. For control of binding specificity, the reactions were performed with both an excess of cold probe and specific N-MYC antibody (Calbiochem). For negative controls, no antibody or non-specific antibody (Myc tag antibody, Abcam) was utilized for immunoprecipitation. The immunoprecipitated complexes were collected with salmon sperm DNA/Protein A-agarose slurry (Upstate) after 1 h of rotation at 4 °C. The collected Protein A-agarose-antibody-chromatin complex was washed three times with Low Salt Immune Complex Wash Buffer (Chromatin Immunoprecipitation ChIP Assay Kit, Upstate), High Salt Immune Complex Wash Buffer (Chromatin Immunoprecipitation ChIP Assay Kit, Upstate), and Immune Complex Wash Buffer (Chromatin Immunoprecipitation ChIP Assay Kit, Upstate) and two times with Tris-EDTA buffer. The immune complexes were eluted from the antibody by incubating with 1% SDS and 0.1 mM NaHCO3 for 15 min at room temperature two times. To reverse the protein-DNA cross-links the combined eluates were heated with 0.2 M NaCl for 4 h. Samples of input starting DNA were made before the immunoprecipitation step by collecting the sonicated cell supernatant fraction in ChIP Dilution Buffer and reversing protein-DNA cross-links by adding 0.3 M NaCl and incubating for 4 h at 65 °C. DNA was purified using phenol-chloroform extraction and ethanol extraction and used for PCR analysis. PCR analysis was performed to identify the binding of N-MYC to the FAK promoter with the following primers: forward, 5′-TCACTTCTGTCTAAAGCCC-3′; and reverse, 5′-GGGACTTAGAAGTCCTGAG-3′. As a control, ChIP assay was also performed with the published telomerase promoter sequence (hTERT), and PCR was performed with the following primers: forward, 5′-AGTTGGATTGCAGCCGACAGA-3′; and reverse, 5′-TTCACCTGGCAGCAGA-3′ (38).

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**siRNA Transfection**—Cells were plated on 96-well culture plates at a density of 5 × 104 cells/well and allowed to attach for 24 h. RNA interference for FAK included FAK siRNA
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(SMARTpool FAK reagent, Dharmacon, Inc.) and the following specific siRNA sequences to FAK: D-05 FAK siRNA, 5’-GAA-GUUGGGUGUCAGAUAU-3’; and D-07 FAK siRNA, 5’-GGAAAUUGCUUGAAGUUGU-3’. Control siRNA included siCONTROL nontargeting siRNA (Dharmacon, Inc.) and GAPDH siRNA (SMARTpool GAPDH reagent, Dharmacon, Inc.). The cells were transfected with either FAK or control siRNA at 0.14 μM with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Cells were incubated 48–72 h after transfection and then used for experiments. FAK inhibition by siRNA was verified with Western blotting with FAK antibodies.

Adenoviral Infections—Cells were plated at 5 × 10^3 cells/well into a 96-well culture plate and allowed to attach for 24 h. The cells were then infected with control AdGFP, AdLacZ, or dominant-negative FAK, AdFAK-CD (12). The AdFAK-CD (analogous to FAK-related non-kinase) construct is an adenoviral construct that contains the carboxyl-terminal domain of FAK (FAK-CD). AdFAK-CD has been described previously in detail by Xu et al. (12). FAK-CD is analogous to the FAK-related non-kinase molecule, which is known to decrease the phosphorylation of p125FAK. Adenoviral transduction of FAK-CD causes loss of cellular adhesion and viability and the loss of p125FAK from focal adhesions (12). Optimal concentrations of virus were determined using AdGFP as described previously (12, 40). We used viral titers that caused expression of green fluorescent protein in 100% of the cells without any toxic effect and was equal to 500 fluorescent focus-forming units/cell as described previously (14, 27, 40, 41). Cells were used 48 h after infection for further experiments.

Cell Viability Assays—Cellular viability was measured with both Cell Titer 96 AQeous One solution assay kit (Promega) and trypan blue exclusion. In brief, cells were plated at 5 × 10^3 cells/well on 96-well culture plates and allowed to attach. Cells were treated with control siRNA, FAK siRNA, or adenoviruses as described above. For the siRNA experiments, after 72 h of transfection, 20 μl of Cell Titer 96 AQeous One solution reagent was added to 100 μl of cell medium. After 1–4 h, the absorbance at 490 nm was measured using a microplate reader (Vmax, Molecular Devices). Cellular viability after adenoviral infections was measured as above after 48 h of infection. For trypan blue exclusion studies, cells were treated with control siRNA, FAK siRNA, or adenoviruses as described above, and trypan blue was added. Stained and unstained cells were counted with a hemacytometer with viability expressed as -fold change in viable cells.

Apoptosis Assays—Cellular apoptosis was evaluated with either a fluorescence-activated cell sorter or Hoechst staining. Following treatment of cells with siRNA or adenoviruses as described above, the cells were treated according to the manufacturer’s instructions and stained utilizing an APO-BRDU kit (BD Pharmingen). Stained cells were analyzed with a FACS-Calibur flow cytometer (BD Biosciences). Calculation of the percentage of apoptotic cells in the sample was completed with CellQuest software (BD Biosciences). Apoptosis was also evaluated with Hoechst 33258 (Sigma) staining as described previously (14). Cells were treated with siRNA or adenoviruses as described above. Briefly cells were collected and fixed in 3.7% formaldehyde in 1× phosphate-buffered saline solution. Hoechst 33258 in 1× phosphate-buffered saline solution (1 μg/ml) was added to the fixed cells for 10 min, and cells were washed twice with 1× phosphate-buffered saline solution and examined with a fluorescence microscope. The percentage of apoptotic cells was calculated on a blinded basis as a ratio of cells with fragmented condensed nuclei/total number of cells with nonfragmented, noncondensed nuclei in three independent experiments. Over 300 cells/sample were analyzed.

RESULTS

Neuroblastoma Cell Lines with Increased N-MYC Expression Up-regulate FAK mRNA and Protein Levels—To determine whether endogenous N-MYC regulates expression of FAK, we used real time RT-PCR to examine FAK mRNA abundance in neuroblastoma cell lines with documented differences in N-MYC amplification. We utilized SK-N-AS human neuroblastoma cells that do not have amplified N-MYC and have low expression of N-MYC protein (32, 33) and compared them to N-MYC-amplified IMR-32 neuroblastoma cells that have significant N-MYC protein expression (33, 34). These two cell lines, although they are not isogenic, were chosen initially for two reasons: because they have been shown to demonstrate significant differences in growth characteristics (33) and also because of their differences in N-MYC. We found that the N-MYC-amplified IMR-32 cell line has more than an 8-fold increase in FAK mRNA compared with the nonamplified SK-N-AS cells (Fig. 1A). To compare FAK mRNA abundance in an isogenic cell line with different N-MYC expression, we used a tetracycline-repressible N-MYC cell line (33–35). When this cell line is grown in the absence of tetracycline, the N-MYC expression vector is activated, leading to N-MYC expression in the cells. Real time RT-PCR in this cell line showed that FAK mRNA abundance is increased 8-fold in the N-MYC positive cells, N-MYC(+) (Tet-), versus the N-MYC(-) (Tet+) cells, similar to the findings with the N-MYC-amplified IMR-32 cell line (Fig. 1B). Thus, FAK mRNA is more abundant in cells with high levels of N-MYC.

To determine whether FAK protein level in neuroblastoma cell lines is dependent upon N-MYC level, we used immunoblotting in the following neuroblastoma cell lines: SK-N-AS, no N-MYC amplification; IMR-32, amplified N-MYC (Fig. 1C); SK-N-AS cells transiently transfected with an N-MYC expression vector (Fig. 1D); and isogenic N-MYC(-/-) (Tet+/-) cells (Fig. 1E). The N-MYC-amplified IMR-32 cells had a significantly higher expression of FAK protein compared with the unamplified SK-N-AS cells (Fig. 1C). Furthermore transient transfection of an N-MYC expression plasmid into SK-N-AS cells resulted in increased FAK protein expression by this cell line compared with the non-transfected SK-N-AS cells (Fig. 1D). In the stably transfected tetracycline-repressible N-MYC cell line, the expression of both N-MYC and FAK protein was increased in the N-MYC(+) (Tet-) cells compared with the N-MYC(-) (Tet+) cells (Fig. 1E). Thus, high levels of N-MYC correlated with high FAK mRNA and protein levels.

N-MYC Regulates the FAK Promoter—To determine whether the increase in FAK mRNA induced by N-MYC was the result of regulation at the promoter level, we performed
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We have cloned and characterized the FAK promoter previously (21). Different serial deletion constructs of the FAK promoter, described in detail by Golubovskaya et al. (21), were used for the dual luciferase assays in neuroblastoma cell lines with differing N-MYC expression (Fig. 2A). First we performed dual luciferase assays with the FAK promoter constructs in SK-N-AS (nonamplified N-MYC) and IMR-32 (amplified N-MYC) neuroblastoma cells and found significant increases in FAK promoter activity in the IMR-32 (N-MYC-amplified) cell line. A significant increase in promoter activity was detected first with the P-280 construct; less significant increases in promoter activity were detected with the P-564 and P-723 constructs and also with the P-1020 and P-1173 constructs in the IMR-32 cells compared with only a background levels in the SK-N-AS cells (Fig. 2B). Because we found the first significant increase in promoter activity with the P-280 construct and also an increase with the largest construct, P-1173, we utilized these two constructs in the next group of experiments to evaluate the direct N-MYC effects on FAK promoter activity in SK-N-AS cells that were transiently transfected with the N-MYC expression plasmid as described above (Fig. 1D). The N-MYC transiently transfected SK-N-AS cells demonstrated a significant increase in promoter activity with the P-280 construct (Fig. 2C). The increased promoter activity in the transiently N-MYC-transfected SK-N-AS cells was most prominent with the P-280 construct compared with the P-1173 construct, similar to the SK-N-AS cells transiently transfected with the N-MYC plasmid (not shown). The increased activity of the P-280 construct in the N-MYC transiently transfected SK-N-AS cells compared with the activity of the P-1173 construct suggests that P-280 contains the critical region for N-MYC binding.

Thus, to examine the specific effect of N-MYC on FAK promoter activity, we examined FAK promoter activity in the isogenic, stably transfected, N-MYC repressible (Tet-off) cell line (Fig. 1E). These sets of experiments were performed to show that the P-280 construct contains the critical region with the N-MYC sites. The N-MYC+ (Tet−) cells demonstrated a significant increase in FAK promoter activity with the P-280 construct compared with the N-MYC− (Tet+) cells (Fig. 2D). Importantly the promoter activity with the P-280 construct was higher than that with the P-1173 construct in this isogenic cell line, similar to the SK-N-AS cells transiently transfected with the N-MYC plasmid (Fig. 2, C and D). Thus, these results clearly show that N-MYC increases FAK promoter activity and indicate that

![Graph](image_url)

**FIGURE 1.** Neuroblastoma cell lines with increased N-MYC expression show an increase in FAK mRNA abundance and p125<sup>FAK</sup> expression. A, fold change in FAK mRNA abundance in SK-N-AS human neuroblastoma cells (nonamplified N-MYC) and IMR-32 human neuroblastoma cells (amplified N-MYC) analyzed by real-time RT-PCR with cyclophilin A as a control. B, fold change in FAK mRNA abundance in the isogenic N-MYC<sup>−/−</sup> (Tet<sup>−</sup>) cell lines analyzed by real-time RT-PCR with cyclophilin A as a control. The cell lines that overexpress N-MYC (both endogenously and through stable transfection) have an 8-fold increase in FAK mRNA abundance. Data are reported as means ± S.E. C, D, and E, representative Western immunoblots of N-MYC and p125<sup>FAK</sup> proteins in the nonamplified SK-N-AS and N-MYC-amplified IMR-32 human neuroblastoma cell lines (C), SK-N-AS cell line transiently transfected for 48 h with N-MYC expression vector (D), and isogenic N-MYC<sup>−/−</sup> (Tet<sup>−</sup>) human neuroblastoma cell lines incubated with and without tetracycline (1 μg/ml), respectively, for 48 h (E). Blots were stripped and reprobed with the indicated antibodies. There is a significant increase in FAK protein expression in all N-MYC-overexpressing cell lines.
the P-280 construct is critical for N-MYC binding to the FAK promoter.

Identification of N-MYC Binding Sites within the FAK Promoter—Next we performed an analysis of the potential N-MYC binding sites in the FAK promoter sequence (21). The N-MYC protein is a transcription factor that interacts with a DNA sequence known as an E-box sequence (CA(C/T)GTG) (42). This sequence and other known binding motifs such as CGCGTG, CACGCG, CATGCG, and CACGAG have been characterized (39, 43–48). We utilized the TRANSFAC

![Diagram of FAK promoter constructs](image1)

A, Fragment Length

| Construct Name | -1173 | -1020 | -723 | -564 | -280 | -109 | -50 | P-Basic |
|---------------|-------|-------|------|------|------|------|-----|---------|
| LUC           | +47   |       | +47  | +47  | +47  | +47  | +47 |         |

B, representative graph showing fold change in FAK promoter activity in SK-N-AS (nonamplified N-MYC) and IMR-32 (amplified N-MYC) neuroblastoma cells transfected with FAK promoter constructs.

C, representative graph showing fold change in FAK promoter activity in N-MYC−/− (Tet−) cells transfected with P-280 and P-1173 constructs. The luciferase activity is expressed relative to the activity seen with SK-N-AS (nonamplified N-MYC) cells with the P-280 construct and presented as mean fold change ± S.E. In all N-MYC-overexpressing cell lines, promoter activity is significantly increased with the P-280 construct. The isogenic N-MYC+/− (Tet−) cells have significantly less promoter activity with the P-1173 construct than with the P-280 construct, showing that the P-280 region is critical with its N-MYC binding sites.
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To specifically demonstrate the role of N-MYC transcription factor in the regulation of FAK promoter activity, we deleted the N-MYC binding site from the FAK promoter construct to compare its activity with the wild-type site in a dual luciferase assay. Because our previous studies showed that the important N-MYC binding site was site 2, we utilized site-specific mutagenesis to alter the P-280 luciferase construct with deletions of 6 base pairs of the putative N-MYC 2 binding region (ΔP-280) to compare it with the wild-type P-280 construct. There was a significant decrease in luciferase activity with the ΔP-280 plasmid containing the mutated site compared with the activity with the wild-type site construct, P-280, in the N-MYC (Tet−) cell line (Fig. 2F). The residual basal activity of the ΔP-280 construct in the N-MYC (Tet−) cells did not differ from the background activity of the P-280 construct in the N-MYC (Tet+) cells (Fig. 2F). Thus, these results clearly demonstrate that N-MYC specifically induces FAK promoter activity through binding to the FAK promoter.

N-MYC Binds to the FAK Promoter—To determine whether N-MYC directly binds to the FAK promoter in vivo, we used a ChIP method with a monoclonal antibody against N-MYC (Fig. 3) utilizing the N-MYC repressible Tet-off cells that had been incubated with and without tetracycline for at least 72 h to completely minimize the expression of N-MYC (Fig. 4A). In N-MYC+ cells grown in the absence of tetracycline at 72 h (N-MYC+, Tet−), significant precipitation of the FAK promoter was seen with the specific N-MYC antibody (Fig. 3, Lane 6) and not seen in the absence of antibody or with the control antibody (Myc tag) (Fig. 3, Lanes 4 and 5). In contrast, in N-MYC− cells grown with tetracycline at 72 h and expressing a very low background level of N-MYC (N-MYC−, Tet+) the ChIP assay was negative as no detectable signal was generated in immunoprecipitation either with the N-MYC-specific antibody, with the control antibody (Myc tag), or in the absence of antibody (Fig. 3, Lanes 9–11). In addition, the same results were obtained with the published telomerase (hTERT) promoter (35) with N-MYC binding in the N-MYC (Tet−) cells and absence of binding in the N-MYC (Tet+) cells (data not shown). Thus, these ChIP studies clearly show that N-MYC binds to the FAK promoter nucleoprotein complex in vivo.
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![Diagram of N-myc+ (Tet-) cells and N-myc- (Tet+) cells with FAK Promoter](Image)

**FIGURE 3. ChIP assay demonstrates N-MYC binding to the FAK promoter in vitro.** ChIP assay detects N-MYC protein bound to the FAK promoter. Isogenic N-MYC-/- (Tet-) cells were incubated with and without tetracycline, respectively, for 72 h resulting in almost complete inhibition of N-MYC expression in the (Tet-) cells (Fig. 4A). The cells were utilized, and protein-DNA complexes were precipitated with either no antibody, nonspecific antibody (Myc tag), or specific N-MYC antibody. PCR amplification was performed using FAK promoter primers. A positive signal was seen with the anti-N-MYC antibody in the N-MYC+ (Tet-) cells (Lane 5) but not in the absence of antibody or with the nonspecific (Myc tag) antibody (Lanes 4 and 5). No signal was generated in the N-MYC+ (Tet-) cells immunoprecipitated with no antibody, nonspecific (Myc tag) antibody, or anti-N-MYC antibody (Lanes 9–11). PCRs with input and genomic DNA were used as positive controls (Lanes 2 and 3 and Lanes 7 and 8).

Furthermore to confirm binding between N-MYC and the FAK promoter in vitro, we utilized EMSA. We performed EMSA with oligonucleotides that were synthesized to contain either an N-MYC consensus binding sequence or the putative N-MYC binding sites on the FAK promoter, N-MYC 1 and N-MYC 2 (Fig. 2E). Nuclear extracts from human neuroblastoma cell lines with minimal, SK-N-AS and N-MYC-/- (Tet-) (31–33), and high, SK-N-BE(2) and Kelly, N-MYC expression (34) were utilized (Fig. 4A). Nuclear extracts from N-MYC-overexpressing SK-N-BE(2) neuroblastoma cells formed gel shift complexes when incubated with the biotin-labeled oligonucleotide for the consensus, N-MYC 1, and N-MYC 2 sites (Fig. 4B, Lanes 2, 5, and 7) indicating binding of N-MYC to FAK promoter probes. In addition, preincubation of the SK-N-BE(2) nuclear lysate with the consensus site in the presence of N-MYC-specific antibody resulted in inhibition of specific complex formation with a supershift of the complex (Fig. 4B, Lane 3). Competition experiments were performed by incubating the SK-N-BE(2) nuclear extracts with excess cold oligonucleotides for consensus, N-MYC 1, and N-MYC 2 and excess N-MYC-specific antibody to establish specificity of N-MYC binding. Inhibition of complex formation was seen with increasing excess cold probe with the consensus (Fig. 4C, Lanes 1–4) and with increasing excess cold probe and N-MYC antibody with N-MYC 1 (Fig. 4C, Lanes 5–9) and N-MYC 2 probes (Fig. 4C, Lanes 10–14). In addition, we performed EMSA with two N-MYC-negative or very low expressing neuroblastoma cell lines (Fig. 4A) and compared them with an N-MYC-overexpressing SK-N-BE(2) neuroblastoma cell line (Fig. 4D). Importantly there was significant binding of N-MYC to the FAK promoter in the N-MYC-expressing cell line, SK-N-BE(2) (Fig. 4D, Lanes 4 and 8), that was absent in the N-MYC-nonexpressing cell lines (Fig. 4D, Lanes 2, 3, 6, and 7). The same results were seen with nuclear extracts from N-MYC-expressing Kelly neuroblastoma cells (data not shown). Therefore, the EMSA and ChIP results clearly show that N-MYC transcription factor specifically binds to the FAK promoter in vitro and in vivo, respectively.

**FAK Down-regulation Decreases Cellular Viability in N-MYC-expressing Cells**—To examine the functional significance of increased FAK expression in N-MYC-expressing neuroblastoma cell lines, we analyzed cell growth of Tet-repressible N-MYC+-/- isogenic cell lines. It is known that N-MYC-amplified neuroblastomas are more aggressive in vivo and tend to grow more rapidly in vitro (6–9, 49). Using phase-contrast microscopy, we showed differences in cellular growth over time between the N-MYC+ (Tet+) and N-MYC- (Tet-) cell lines (Fig. 5A). We also utilized cell viability assays to demonstrate increased viability in the N-MYC+ (Tet+) cells over time (Fig. 5, B and C). To demonstrate that down-regulation of FAK expression decreases cellular viability in the N-MYC+ (Tet+) cells, we inhibited FAK expression with either different FAK siRNAs or a dominant-negative inhibitor of FAK (AdFAK-CD) (12, 14, 27, 41). Inhibition of FAK with siRNA significantly decreased the level of FAK in the N-MYC- (Tet-*) (Fig. 6A) and N-MYC+ (Tet-) (Fig. 6B) cells, whereas control siRNA did not decrease FAK levels (Fig. 6, A and B). Because FAK expression was initially higher in the N-MYC+ (Tet-) cells compared with the N-MYC- (Tet*) cells (Fig. 1E), we tested the sensitivity of the cells to down-regulation of FAK. Inhibition of FAK expression with two different FAK siRNAs in the N-MYC- (Tet-) cells with low FAK expression resulted in no significant changes in cell viability compared with the untreated and control siRNA-treated cells (Fig. 6, C and D). Importantly FAK siRNA treatment of the N-MYC+ (Tet-) cells with higher initial FAK expression than the N-MYC- (Tet-) cells (Figs. 1E and 6, A and B) resulted in a significant decrease in cell viability compared with the untreated and control siRNA-treated N-MYC+ (Tet-) cells (Fig. 6, C and D). The use of a dominant-negative FAK inhibitor, AdFAK-CD, also produced similar results. Inhibition of FAK expression with AdFAK-CD in the N-MYC+ (Tet-), high FAK-expressing cells resulted in significantly decreased cellular viability when compared with the control groups (Fig. 6E). In contrast, in N-MYC- (Tet*) cells with low FAK expression the difference in viability was not significant (Fig. 6E). Therefore, N-MYC+ (Tet-) cells with higher expression of FAK were significantly more sensitive to FAK down-regulation than the N-MYC- (Tet*) cells with lower initial FAK expression. These data are consistent with data on breast cancer cell lines where cells with high FAK expression were more sensitive to AdFAK-CD than low FAK-expressing cells (12). Therefore, these findings clearly demonstrate that FAK down-regulation decreases cellular viability in N-MYC+ (Tet-) cells.

**FAK Down-regulation Increases Cellular Apoptosis in N-MYC-expressing Cells**—To further examine the biologic significance of increased FAK expression in N-MYC-expressing neuroblastoma cell lines, we analyzed cellular apoptosis in the isogenic N-MYC-/- (Tet+/−) neuroblastoma cell lines after FAK inhibition with siRNA or AdFAK-CD. There was a significant increase in the percentage of apoptosis...
FIGURE 4. N-MYC binds to the FAK promoter in vitro by EMSA. A, representative Western immunoblot for N-MYC protein in nuclear extracts from differing N-MYC-expressing neuroblastoma cell lines. There is minimal N-MYC expression in the N-MYC-nonexpressing SK-N-AS and N-MYC \(^{-}\) (Tet \(^{-}\), 72 h) nuclear extracts compared with the N-MYC-amplified SK-N-BE(2) extracts. B, EMSA showing nuclear extracts from SK-N-BE(2), N-MYC-overexpressing neuroblastoma cells. There is significant N-MYC and FAK promoter binding when the extracts are incubated with biotin-labeled consensus, N-MYC 1, or N-MYC 2 oligonucleotides (Lanes 2, 5, and 7). The addition of 0.1 \(\mu\)g of N-MYC antibody with the consensus oligonucleotides results in supershift (Lane 3). C, EMSA showing competition experiments with nuclear lysates from N-MYC-overexpressing SK-N-BE(2) cells. With the addition of increasing amounts of cold probe (× 100 and ×1000) with the biotin-labeled consensus, N-MYC 1, and N-MYC 2 oligonucleotides, binding of N-MYC to the FAK promoter is inhibited (Lanes 2–4, 6–8, and 11–13, respectively). The addition of 0.1 \(\mu\)g of N-MYC antibody with the N-MYC 1 and N-MYC 2 oligonucleotides results in inhibition of N-MYC binding to the FAK promoter (Lanes 9 and 14). D, EMSA showing that there is no N-MYC binding to FAK promoter with N-MYC-nonexpressing neuroblastoma cell lines, SK-N-AS and N-MYC \(^{-}\) (Tet \(^{-}\), 72 h) (Lanes 2 and 3 and Lanes 6 and 7). Ab, antibody.
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Inhibition of FAK in the N-MYC<sup>-</sup> (Tet<sup>-</sup>) cells (Fig. 7, A and B). Also following FAK inhibition in the N-MYC<sup>+</sup> (Tet<sup>-</sup>) cells with a dominant-negative FAK (AdFAK-CD), there was a significant (>4-fold) increase in percentage of apoptotic cells compared with control treated cells or those cells treated with control AdLacZ (Fig. 7C). The N-MYC<sup>-</sup> (Tet<sup>-</sup>) cells were not significantly affected by FAK inhibition with AdFAK-CD (Fig. 7C). Apoptosis with FAK siRNA and AdFAK-CD was confirmed by poly(ADP-ribose) polymerase and caspase 8 and caspase 3 activation (not shown). These findings demonstrate the biologic significance of FAK expression in the N-MYC<sup>-</sup> (Tet<sup>-</sup>) cells and the significance of N-MYC regulation of FAK in these cells.

**DISCUSSION**

Focal adhesion kinase has been shown to be overexpressed in a number of human tumors (15, 16, 50) including colon (9, 17, 19, 20), breast (17, 19), ovarian (51), and thyroid cancers (18). In the current study, we showed that FAK is overexpressed in human neuroblastoma cell lines with N-MYC overexpression. Other investigators have shown an increased abundance of FAK mRNA in human colon and breast tumors correlating with increased FAK protein expression in these same tumors (20, 21), indicating a transcriptional regulation of FAK. Therefore, we undertook this study to analyze N-MYC-regulated FAK expression in neuroblastoma cell lines.

In this study, we identified N-MYC as a novel transcription factor for the focal adhesion kinase gene in neuroblastoma. We demonstrated by real time PCR and Western immunoblot assays that FAK mRNA and protein levels are increased in neuroblastoma cell lines with increased N-MYC levels. We demonstrated that neuroblastoma cells with endogenous N-MYC amplification, transiently transfected and overexpressing N-MYC, and stably transfected and overexpressing N-MYC show increased FAK promoter activity. N-MYC transcription factor activated a promoter/luciferase reporter containing two N-MYC binding sites. Site-directed mutagenesis of the putative N-MYC binding site resulted in a significant decrease in FAK promoter activity compared with the construct with the wild-type binding site. These results clearly indicate that FAK is a novel target gene for N-MYC in neuroblastoma.

Previous studies have documented significantly increased motility, growth, and survival in N-MYC-amplified versus nonamplified neuroblastoma cell lines (6–10, 49). Non-isogenic cells can have cell type-specific differences in gene regulation. For example, SK-N-AS cells transfected with an N-MYC plasmid did not have an increase in activity of the P-1173 construct (Fig. 2C) as was seen with the N-MYC-amplified IMR-32 cells (Fig. 2B). We also found that the N-MYC-amplified SK-N-BE(2) and SK-N-DZ cell lines had a
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significant increase in activity of the P-280 construct but no increase in the P-1173 construct. These findings suggest cell type-specific differences and the presence of other transcription binding sites that can either down-regulate, as in P-564 and P-723 constructs (Fig. 2B), or up-regulate, as in P-1173 construct, (Fig. 2B) promoter activity in IMR-32 cells. Therefore and most importantly for these studies, we used an isogenic model of human neuroblastoma cells that are not N-MYC-amplified (SHEP) (33, 34) and have been stably transfected with an N-MYC tetracycline-repressible expression vector. The N-MYC<sup>-/-</sup> (Tet<sup>-</sup>) cell line has been well characterized (35), and the cells express minimal amounts of N-MYC in the presence of tetracycline. However, in the absence of tetracycline, the N-MYC expression vector is activated, and their N-MYC expression increases significantly. We used the N-MYC<sup>-/-</sup> (Tet<sup>-/-</sup>) isogenic cell line in our study to minimize cell type-specific differences, and we demonstrated significant activation of the FAK promoter in the N-MYC<sup>+</sup> (Tet<sup>-</sup>) cells compared with the N-MYC<sup>-</sup> (Tet<sup>+</sup>) cells.

N-MYC transcription factor has been shown to modulate gene expression through binding to a set of E-box motifs that possess a core consensus sequence of CA(C/T)GTG (43, 45). In this study, our deletion experiments showed that N-MYC binds to the CATGCG variant E-box site in the FAK promoter and induces transcription of the FAK gene. This finding is consistent with a number of reported studies. Blackwell et al. (52) and Cohn and co-workers (48) found that N-MYC protein could bind with numerous variant E-box sites including CATGCG, and other investigators have demonstrated that N-MYC binds to numerous gene promoters in the face of other E-box sequences (39, 46). Our current study used three different methods including luciferase promoter-reporter assays, ChIP, and EMSA assays to demonstrate N-MYC binding to the FAK promoter. Our ChIP assay and EMSA studies clearly showed an absence of N-MYC binding in the N-MYC<sup>-/-</sup> (Tet<sup>-/-</sup>) cells. Some

FIGURE 6. Down-regulation of FAK with siRNA or AdFAK-CD decreases cellular viability in N-MYC-expressing cells. A, Western blotting was utilized to show decreased FAK protein expression in N-MYC<sup>-</sup> (Tet<sup>-</sup>) cells after treatment with FAK siRNA. Lipofectamine alone (Control) or control siRNA does not significantly affect the FAK protein expression in these cells. B, Western blot demonstrating a significant decrease in FAK protein expression in the N-MYC<sup>+</sup> (Tet<sup>-</sup>) cells after transfection with FAK siRNA but not after transfection with Lipofectamine alone (Control) or control siRNA. FAK pooled siRNA is shown. The same FAK inhibition was observed with other FAK siRNAs (‘Experimental Procedures’). C, FAK inhibition was induced with FAK siRNA. FAK (pooled) siRNA is shown. There is a significant decrease (p < 0.05) in cellular viability in N-MYC<sup>-</sup> (Tet<sup>-</sup>) cells with high initial FAK expression (Fig. 1E) by FAK siRNA (pooled) but not by GAPDH siRNA (Control siRNA) or cells treated with Lipofectamine alone (Control). There is no significant difference in cellular viability of N-MYC<sup>-</sup> (Tet<sup>-</sup>) cells with low initial FAK expression (Fig. 1E) after transfection with control GAPDH siRNA or FAK siRNA, D, additional FAK siRNA, FAK2 (D-07, ‘Experimental Procedures’), was utilized for FAK inhibition. There is a significant decrease (p < 0.05) in cellular viability in N-MYC<sup>-</sup> (Tet<sup>-</sup>) cells with FAK siRNA (D-07) but not with control siRNA (nontargeting control siRNA). There is no significant difference in cellular viability of N-MYC<sup>-</sup> (Tet<sup>-</sup>) cells with FAK siRNA or control siRNA. E, FAK inhibition was also induced with a dominant-negative FAK, AdFAK-CD. Cellular viability was measured in N-MYC<sup>+/+</sup> (Tet<sup>-/-</sup>) cell lines after infection with AdLacZ or AdFAK-CD. There is a significant decrease in viability in the N-MYC<sup>-</sup> (Tet<sup>-</sup>) cells with AdFAK-CD, but these cells are not affected by control AdLacZ. The N-MYC<sup>-</sup> (Tet<sup>-</sup>) cells are unaffected by AdFAK-CD, similar to the findings with siRNA treatment (C and D). Cell viability assays were repeated at least three times with data reported as means ± S.E.
**N-MYC Regulates FAK Expression**

**A**

![Image of representative slides of Hoechst staining of N-MYC expressing cells](Image)

**B**

![Graph showing apoptosis](Image)

**C**

![Bar graph showing apoptosis](Image)

**FIGURE 7. Down-regulation of FAK with siRNA or AdFAK-CD increases cellular apoptosis in N-MYC-expressing cells.** A, fluorescence microscopy of representative slides of Hoechst staining of N-MYC\(^{+/+}\) (Tet\(^{-}\)) neuroblastoma cells following treatment with two FAK siRNAs (1 and 2). Apoptotic cells with condensed and fragmented nuclei are demonstrated by the white arrows. B, N-MYC\(^{+/+}\) (Tet\(^{-}\)) cells were treated with Lipofectamine (Control), GAPDH siRNA, control siRNA (nontargeting control siRNA), or FAK siRNAs (FAK1, D-05; FAK2, D-07). There is a significant increase \((p < 0.05)\) in the percentage of apoptotic cells as detected by Hoechst staining following inhibition of FAK with siRNA in the N-MYC\(^{+/+}\) (Tet\(^{-}\)) cells compared with control treated cells (Lipofectamine alone) and those treated with GAPDH or control siRNA. In contrast, apoptosis in the N-MYC\(^{-/-}\) (Tet\(^{-}\)) cells was not affected by siRNA treatment. C, FAK inhibition was induced in N-MYC\(^{-/-}\) (Tet\(^{-}\)) cells with AdFAK-CD. By fluorescence-activated cell sorter analysis, there is a significant \((p < 0.01)\) increase in percentage of apoptotic cells in the N-MYC\(^{-/-}\) (Tet\(^{-}\)) cells after treatment with AdFAK-CD compared with untreated cells and those treated with AdLacZ. All apoptosis assays were repeated at least three times with data reported as means \(\pm S.E.\)

N-MYC binds to the FAK promoter, which is shown in the ChIP assay in the N-MYC\(^{-/-}\) (Tet\(^{+}\)) cells suggests the presence of other transcription factors present in these cells that can up- or down-regulate the promoter. Importantly, we found that the promoter activity was significantly increased by the introduction of N-MYC into the cells, and the activity was significantly decreased by mutating the N-MYC binding site. Therefore, these studies clearly demonstrate, for the first time, that N-MYC binds to the FAK promoter.

The novel findings that N-MYC binds to the FAK promoter in vitro and in vivo show that the expression of FAK is regulated by N-MYC in neuroblastoma cells. Further translational studies with primary tumors will need to be completed to determine the possible contribution of FAK as both a prognostic indicator and upon tumor pathogenesis and, ultimately, patient survival in human neuroblastoma.

The transcriptional targets of N-MYC are of considerable interest in cancer and especially in neuroblastoma because of the association of extremely poor prognosis of this tumor with amplification of N-MYC oncogene (4). It is hypothesized that N-MYC targets are related to improved cellular proliferation and resistance to apoptosis and cellular differentiation (53–55). Focal adhesion kinase has been well characterized in a number of human tumor types (15, 16, 50), but this is one of the few studies reporting the up-regulation of this kinase in human neuroblastoma. There is mounting evidence that FAK is involved in cellular survival functions (12, 26, 56–59). Our results show that N-MYC induction of FAK provides a biologic advantage to the neuroblastoma cells. N-MYC\(^{+/+}\) (Tet\(^{-}\)) cells with higher FAK expression were more susceptible to FAK down-regulation and had decreased viability and increased apoptosis following FAK inhibition with siRNA or AdFAK-CD compared with the lower FAK-expressing N-MYC\(^{-/-}\) (Tet\(^{-}\)) cells. Similar results have been reported with breast cancer cell lines showing that those cell lines with increased FAK expression are more affected by FAK blockade than those cells with lower FAK expression (12). Thus, N-MYC binding and activation of the FAK promoter may serve as a mechanism of regulating cellular viability and apoptosis in neuroblastoma.

We found a difference in the degree of decreased cell viability and increased apoptosis in response to FAK inhibition with siRNA and AdFAK-CD. The N-MYC\(^{+/+}\) (Tet\(^{-}\)) neuroblastoma cells were more affected by FAK abrogation with AdFAK-CD than with siRNA. Our data are comparable to that of other authors who have noted similar results when attempting to directly compare effects of FAK knockdown with siRNA to FAK-related non-kinase (FAK dominant-negative) (60, 61). However, our results with FAK inhibition with both methods clearly demonstrate a biologic advantage to N-MYC regulation of FAK.

Our results demonstrating the effects of N-MYC upon the FAK promoter in neuroblastoma may impact our understanding of FAK biology and tumorigenesis in other tumor types. N-MYC is reported to be amplified in human melanoma and sarcomas (62) and is associated with poor outcomes (63). It has also been shown that FAK is overexpressed in human sarcoma (16) and melanoma tumors (64, 65) and that down-regulation of FAK results in decreased survival in...
human melanoma cells (66). The regulation of FAK expression by N-MYC in these tumor types has not been investigated but may contribute to the molecular regulation of FAK in these tumors.

The results of this study support a novel role for N-MYC in the regulation of FAK expression in neuroblastomas. This is the first study to directly link the interaction of the transcription factor N-MYC with FAK, and it provides a basis for a number of future studies examining the effects of N-MYC and FAK expression and signaling on cellular survival.

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