Two N-Myristoyltransferase Isozymes Play Unique Roles in Protein Myristoylation, Proliferation, and Apoptosis

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Abstract
N-myristoyltransferases (NMT) add myristate to the NH₂ termini of certain proteins, thereby regulating their localization and/or biological function. Using RNA interference, this study functionally characterizes the two NMT isozymes in human cells. Unique small interfering RNAs (siRNA) for each isozyme were designed and shown to decrease NMT1 or NMT2 protein levels by at least 90%. Ablation of NMT1 inhibited cell replication associated with a loss of activation of c-Src and its target FAK as well as reduction of signaling through the c-Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase kinase/extracellular signal-regulated kinase pathway. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assays showed that depletion of either NMT isozyme induced apoptosis, with NMT2 having a 2.5-fold greater effect than NMT1. Western blot analyses revealed that loss of NMT2 shifted the expression of the BCL family of proteins toward apoptosis. Finally, intratumoral injection of siRNA for NMT1 or for both NMT1 and NMT2 inhibited tumor growth in vivo, whereas the same treatment with siRNA for NMT2 or negative control siRNA did not. Overall, the data indicate that NMT1 and NMT2 have only partially overlapping functions and that NMT1 is critical for tumor cell proliferation.

Introduction
The mature forms of many proteins contain various covalent modifications, including lipids, phosphates, acetyl groups, formyl groups, and nucleotides. The structural changes resulting from these modifications have effects on the stability, cellular location, and biological activity of the proteins (1, 2). These processes are becoming increasingly important for the study of cancer, as several key oncoproteins require this type of “post-translational maturation” for their biological active and for their ability to transform cells (3, 4). The Src family of tyrosine kinases (e.g., c-Src, Yes, and Fyn) is an example of oncogenic molecules that require a myristate moiety attached to their NH₂ terminus in order for them to function in cells. This was first shown in studies showing that the viral oncogene product v-Src requires myristoylation for membrane binding and cellular transformation (5-7). Mutation of the NH₂-terminal myristoylated glycine residue of Src-related tyrosine kinases blocks myristoylation and inhibits the ability of these proteins to transform cells without affecting their intrinsic kinase activity (7, 8).

The roles of the Src family of tyrosine kinases have been explored in several transformed cell lines, and these proteins are central intermediates in conveying both extracellular and intracellular signals to downstream effectors (9, 10). Interestingly, activating mutations in c-src are rare and have only been found in a small subset of advanced colon cancers and in a single endodermal cancer. However, high levels of Src activity are common in cancer as a result of a variety of Src stimulators (11, 12). Once Src activity is increased, it is involved in the migration, proliferation, adhesion, and angiogenesis of the affected cells (11). A large body of evidence has accumulated regarding the expression and activation of Src in several tumor types, including colon, breast, and ovarian tumors. Unfortunately, there is no single activator in these diseases and the transformation of the cells is mediated by an unmutilated cellular protein (c-Src), making it difficult to design a therapeutic agent for their treatment. However, the fact that c-Src is a key regulator of cellular transformation and progression also presents an opportunity for therapeutic manipulation.

Recently, it has been shown that the enzyme myristoyl-CoA-protein N-myristoyltransferase (NMT) that is involved in the post-translational modification of c-Src is also overexpressed in colon tumor cells and human brain tumors (13-17). Further, inhibiting the myristoylation of Src in colon cancer cell lines prevents the localization of the kinase to the plasma membrane and results in decreased colony formation and cell proliferation (18). Because NMTs and Src are overexpressed in colonic tumors (16), NMT inhibitors have the potential to be an important advance in colon cancer therapeutics.

N-myristoylation involves the covalent attachment of myristate, a 14-carbon saturated fatty acid, to the NH₂-terminal glycine residue of specific proteins. NMT is responsible for this activity in eukaryotic cells and works by modifying its polypeptide substrate after the removal of the initiator
methionine residue by methionyl aminopeptidase (19, 20). This modification occurs primarily as a cotranslational process (21, 22), although myristoylation can also occur post-translationally (23-25). Two isozymes of the mammalian NMT enzymes have been cloned and are designated NMT1 and NMT2. The two human NMT enzymes share ~77% identity (26), with the majority of divergence occurring in the NH2-terminal domains. Splice variants of NMT1 have also been observed in some cells. It is thought that these NH2-terminal differences may allow differential cellular localization of the isozymes, thereby allowing either cotranslational ribosome-based or post-translational cytosol-based protein myristoylation. An in vitro comparison of the activity of NMT1 and NMT2 toward a small panel of substrate peptides indicated that the isozymes have similar, but distinguishable, relative selectivity (26, 27). However, there has been few published demonstration of differential functions of NMT isozymes in mammalian cells. In fact, only as this work was coming to a close was there a report published where both NMT1 and NMT2 were considered (28). In this report, the authors conclude that NMT2 is not active in embryonic stem cells but they also show that NMT2 levels increase during development. This report also shows the distribution of both isozymes in normal tissue. Unfortunately, they do not pursue the function of NMT2 either in the fetal mouse or in the pups.

To elucidate the roles of the NMT isozymes in human cells, small interfering RNAs (siRNA) have been used to selectively knockdown the expression of NMT1, NMT2, or both isozymes in human colon cancer HT-29 cells and ovarian carcinoma SK-OV-3 cells. This study reports siRNA sequences unique to each NMT message that selectively reduce the expression of the NMT1 or NMT2 isoyme by >90% for at least 72 hours. With these reagents, we have shown that NMT1 and NMT2 have both redundant and unique effects on protein processing, apoptosis, and cell proliferation. Consequently, these siRNAs provide novel tools to determine the molecular mechanisms by which the individual NMT enzymes function to produce their cellular effects in mature organisms.

Results
Ablation of NMT1 and NMT2 Using siRNAs
In all fungi and nematodes that have been examined, the single NMT enzyme is essential for viability. However, this question has not been addressed for the two human isozymes. Therefore, we sought to selectively abrogate the expression of the two NMT isozymes using siRNA to assess the roles of NMT1 and NMT2 in human cells. Target sites for siRNA constructs for both NMT1 and NMT2 were located and analyzed using the Ambion (Austin, TX) siRNA Target Finder. Of the numerous potential sites in each NMT gene, four were chosen so that they were distributed relatively evenly throughout the coding sequences and were checked to ensure specificity using BLAST searches against the available public databases. Preliminary experiments identified the siRNA constructs that produced the greatest reduction in NMT protein levels (NMT1-1 and NMT2-4), and these reagents were used for subsequent transfection experiments (Fig. 1).

The duration of the knockdown of NMT1 and NMT2 in SK-OV-3 cells is indicated in Fig. 2A. As shown in this Western

![FIGURE 1. siRNA constructs. The sense strand sequences for the NMT1-1 and NMT2-4 siRNAs are shown with the alignment of the cDNA of both enzymes. NMT1-1 represents nucleotides 103 to 124 of NMT1 and NMT2-4 represents nucleotides 1,471 to 1,492 of NMT2.](mcr.aacrjournals.org)
blot analysis, the NMT1-1 siRNA reduced the levels of NMT1 protein to below detectable limits within 24 hours, and the effect persisted for at least 72 hours. The NMT2-4 siRNA reduced the levels of NMT2 protein by ~75% at 24 hours, and complete ablation was seen at 48 and 72 hours. The double knockdown using both siRNAs significantly reduced the levels of both NMT1 and NMT2 at 24 hours and persisted for at least 72 hours. However, it is interesting to note that levels of NMT2 were not fully ablated when NMT1 was absent, suggesting the need to maintain some level of NMT activity. It was also apparent in these experiments that the cells undergoing the double siRNA treatment did not proliferate as fast as those that were untreated and in fact were dying. Figure 2B shows a titration of whole cell lysates probed with either NMT1 or NMT2 antibodies and quantified on the FujiFilm imager. The graph shows the signal intensity of each band versus the concentration of total cell protein loaded for both NMT1 and NMT2 blots.

To quantify the effects of the siRNA treatments, additional experiments were done with total cellular protein and RNA being collected at 48 hours after transfection. In these experiments, images were captured using a CCD imaging system. β-Actin protein and mRNA were used to normalize for small variations in gel loading. Figure 3 shows the quantification of Western blots of total protein from cells treated with 10 nmol/L NMT1-1 siRNA, 10 nmol/L NMT2-4 siRNA, 10 nmol/L each of NMT1-1 and NMT2-4, 20 nmol/L negative control siRNA, and cells treated with no siRNA with and without the transfection reagent, LipofectAMINE 2000, and then probed with antibodies against human NMT1 (Fig. 3A) or NMT2 (Fig. 3B). In cells treated with NMT1-1 siRNA, the levels of NMT1 protein were reduced by 93% and levels of NMT2 protein were increased by 60% compared with negative control siRNA-treated cells or cells treated with only LipofectAMINE 2000. In cells treated with NMT2-4 siRNA, the levels of NMT2 protein were reduced by 95% and levels of NMT1 protein were reduced by 25% compared with negative controls. Finally, in cells treated with both NMT1-1 and NMT2-4 siRNA, levels of NMT1 protein were reduced by 80% and levels of NMT2 protein were reduced by 75% compared with controls. Northern blot analyses were conducted on the same cells and are shown in Fig. 3C. There was an excellent correlation between the mRNA levels of NMT1 and NMT2 and the protein levels observed in the Western blot analyses. Interestingly, NMT2 mRNA levels were increased in the NMT1-1 siRNA-treated cells to the same level as the protein, showing that the increase in NMT2 protein is due to an increase in mRNA levels and not to an increase in protein stability. The Northern blot also allows for an estimation of the relative abundance of NMT1 and NMT2 mRNA in the cell. There was approximately five times more NMT1 message than NMT2 message in untreated (no siRNA and no LipofectAMINE 2000) cells. Effective knockdown of NMT1 and NMT2 in HT-29 cells was achieved with NMT1-1 and NMT2-4 siRNAs; however, 40 nmol/L of each siRNA construct were required for efficient transfection of these cells (data not shown).
GFP to be myristoylated and palmitoylated resulting in its localization in the plasma membrane and that relocalization of GFP to the cytosol occurs when the protein is mutated so that myristoylation cannot occur (31). This reporter construct was used to determine if removal of endogenous NMT1 and/or NMT2 affects the subcellular localization of the modified GFP. A second GFP construct, GFP-farn-palm, containing the COOH-terminal sequence of H-Ras thereby directing it to be farnesylated and palmitoylated, was used as a control because this fusion protein localizes to the plasma membrane in a myristoylation-independent manner. Figure 4 shows SK-OV-3 cells transiently transfected with N-myr-palm-GFP or GFP-farn-palm and NMT1-1 siRNA, NMT2-4 siRNA, both NMT1-1 and NMT2-4, or negative control siRNA. As expected, normal localization of both fusion proteins to the plasma membrane occurred in cells treated with the negative control siRNA. The localization of the N-myr-palm-GFP was not affected by treatment of the cells with either NMT1-1 or NMT2-4 siRNA individually. However, the double knockdown clearly resulted in blockage of GFP localization to the plasma membrane. In contrast, the localization of GFP-farn-palm to the plasma membrane was not affected by either the single knockdowns or the double NMT knockdown. This suggests that NMT1 and NMT2 are each capable of processing this NH2-terminal sequence of Fyn fused to this GFP.

Effects of NMT1 and NMT2 Ablation on Cell Proliferation and Apoptosis

As indicated above, there were always fewer cells in the NMT siRNA-treated cultures than in untreated cultures or cultures treated with the negative control siRNA. To determine the mechanism of the decrease in cell number, cell replication and survival were examined in NMT1-1 and NMT2-4 siRNA-treated cells. First, [3H]thymidine incorporation into DNA was monitored to determine if the knockdowns of NMT1, NMT2, or both had an effect on cell proliferation. In this assay, cells that are actively dividing will incorporate the labeled nucleoside into DNA, whereas cells that are not replicating will not. By measuring the amount of [3H] incorporated into DNA, it is possible to assess the number of cells actively undergoing DNA replication. Equal numbers of SK-OV-3 cells were treated with either 10 nmol/L NMT1-1 siRNA, 10 nmol/L NMT2-4 siRNA, 10 nmol/L each of NMT1-1 and NMT2-4 siRNA, 20 nmol/L negative control siRNA (Neg. Cont.), or no siRNA in the absence and presence of LipofectAMINE 2000 (LF2000) for 48 hours as described in Materials and Methods. Western blots were prepared using 50 μg total protein harvested from cells and were first probed with either anti-NMT1 or anti-NMT2 antibodies, stripped, and then reprobed with a pan-actin antibody. The graphs represent the ratio of NMT1 or NMT2 to actin in each lane. Northern blots were prepared using 10 μg mRNA per lane from the same cell treatments and were probed with NMT1, NMT2, and β-actin probes as described in Materials and Methods.

Effects of NMT1 and NMT2 Ablation on the Localization of N-Myristoylated Green Fluorescent Protein

As Src-related tyrosine kinases (e.g., Fyn) are translated, the NH2-terminal methionine is removed by methionyl aminopeptidase and the newly formed NH2-terminal glycine is N-myristoylated by NMT. Members of this family, other than c-Src, are then palmitoylated on a cysteine residue near the myristoylated NH2 terminus. These modifications are necessary for the maturation and functionality of these proteins because blocking N-myristoylation abolishes their proper localization to the inner leaflet of the plasma membrane (29, 30). We have shown previously that fusing the NH2-terminal sequence of Fyn to green fluorescent protein (GFP; N-myr-palm-GFP) directs GFP to be myristoylated and palmitoylated resulting in its localization in the plasma membrane and that relocalization of GFP to the cytosol occurs when the protein is mutated so that myristoylation cannot occur (31). This reporter construct was used to determine if removal of endogenous NMT1 and/or NMT2 affects the subcellular localization of the modified GFP. As expected, normal localization of both fusion proteins to the plasma membrane occurred in cells treated with the negative control siRNA. The localization of the N-myr-palm-GFP was not affected by treatment of the cells with either NMT1-1 or NMT2-4 siRNA individually. However, the double knockdown clearly resulted in blockage of GFP localization to the plasma membrane. In contrast, the localization of GFP-farn-palm to the plasma membrane was not affected by either the single knockdowns or the double NMT knockdown. This suggests that NMT1 and NMT2 are each capable of processing this NH2-terminal sequence of Fyn fused to this GFP.

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Second, the effects of the siRNAs on the percentage of cells undergoing apoptosis were determined using the terminal deoxynucleotidyl transferase (TdT)–mediated dUTP nick end labeling assay. Cells undergoing apoptosis accumulate...
double-stranded breaks in their DNA and these “free ends” can be labeled with fluorescein-conjugated dUTP through the action of TdT and visualized by microscopy. As above, SK-OV-3 cells were treated with 10 nmol/L NMT1-1 siRNA, 10 nmol/L NMT2-4 siRNA, 10 nmol/L each of NMT1-1 and NMT2-4, or 20 nmol/L negative control siRNA. After 48 hours, the cells were fixed and double-stranded breaks in the DNA were labeled. The experiment was done in triplicate and 15 fields were counted for each slide of three replicates, with the final fold changes representing all 45 fields ± SD. Figure 5 shows representative matched bright-field and fluorescent images of cells treated with NMT1-1, NMT2-4, both NMT1-1 and NMT2-4, or negative control siRNA. The images clearly show that the double knockdown of NMT1 and NMT2 had the most dramatic effect on the cells, with ~30% of the cells undergoing programmed cell death. Interestingly, both NMT1 and NMT2 seem to contribute to this phenotype because the depletion of NMT1 caused an 8-fold increase in apoptosis and the depletion of NMT2 caused a 19-fold increase in apoptosis compared with the control cells.

Effects of NMT1 and NMT2 Ablation on Signaling Proteins

Microarray analyses were done to examine the molecular pathways that NMT1 and NMT2 impact to affect the observed changes in replication and apoptosis. These experiments were done with RNA isolated from HT-29 cells that had been treated with 40 nmol/L NMT1-1, 40 nmol/L NMT2-4, or 40 nmol/L negative control siRNA (data not shown). HT-29 cells were used in these assays because it has been shown that SK-OV-3 cells are not optimal for investigating intracellular signaling due to their constitutive activation of the phosphatidylinositol 3-kinase pathway and their modest response to cellular perturbations (32, 33). The data revealed several interesting findings that were further analyzed in protein expression studies shown in Figs. 6 and 7.

As with the SK-OV-3 cells, the siRNAs NMT1-1 and NMT2-4 promoted selective depletion of NMT1 and NMT2, respectively. The extent of knockdown in the HT-29 cells was slightly lower than in the SK-OV-3 cells; however, the 40 nmol/L doses were sufficient to perturb signaling pathways as described below. In the double knockdown, cellular NMT levels were decreased by >85%. The effects of NMT ablation on the

**FIGURE 4.** Inhibition of N-myristoylated GFP processing by NMT siRNAs. N-myri-palm-GFP and GFP-farn-palm expression constructs were cotransfected into SK-OV-3 cells with the indicated siRNAs as described in Materials and Methods. After 48 hours, the subcellular localization of the GFP was visualized by fluorescence microscopy.
FIGURE 5. Induction of apoptosis by NMT siRNAs. SK-OV-3 cells were treated with 20 nmol/L negative control siRNA (A), 10 nmol/L NMT1-1 siRNA (B), 10 nmol/L NMT2-4 siRNA (C), or 10 nmol/L each of NMT1-1 and NMT2-4 siRNA (D) for 48 hours as described in Materials and Methods and then scored for apoptosis using the TdT-mediated dUTP nick end labeling assay. Panels are matched sets of bright field (bottom) and fluorescent (TdT-mediated dUTP nick end labeling positive; top) images for each treatment. E. Graphical representation of the percentage of apoptotic cells in each treatment condition. Negative control–treated cells had an average of 1.0 ± 0.3% of apoptotic cells. NMT1-1-treated cells had an average of 7.8 ± 1.1% of apoptotic cells. NMT2-4-treated cells had an average of 19.0 ± 2.0% of apoptotic cells. NMT1-1- and NMT2-4-treated cells had an average of 29.8 ± 2.0% of apoptotic cells.
expression of the myristoylated proteins, myristoylated alanine-rich C kinase substrate (MARCKS) and Src, are shown in Fig. 6. Surprisingly, the microarray analyses indicated that c-Src message was up-regulated in the NMT1 knockdown cells compared with the control cells. Western blot analyses of total c-Src protein confirmed that the protein levels are increased in the NMT1 knockdown (Fig. 6). In contrast, levels of activated (i.e., phosphorylated) c-Src were down-regulated in the NMT1 knockdown cells as well as in the double knockdown cells. Because activated c-Src binds to and phosphorylates FAK at residues 576 and 577 (34, 35), the effects of the NMT siRNAs on phospho-FAK levels were also examined. The Western blot in Fig. 6 shows that, when c-Src phosphorylation was reduced by ablation of NMT1, FAK phosphorylation was also reduced. These results suggest that NMT1 is responsible for myristoylation of c-Src and that loss of c-Src myristoylation and activity leads to down-regulation of FAK activity.

A second interesting finding from the microarray analyses was the differential regulation of the MARCKS protein by the knockdown of NMT1 versus NMT2. This protein is of interest for two reasons. First, MARCKS translocation between the plasma membrane and the cytosol (36) has been suggested to mediate destabilization of the cytoskeleton by cross-linking actin filaments at the plasma membrane (37). The activation of MARCKS by phosphorylation and myristoylation has also been shown to be involved in cellular adhesion, cell spreading, and vesicle trafficking, which could play a role in the metastasis of some cancers. Second, the change in total MARCKS protein provides a cellular marker that can be used as a indicator of the inhibition of the NMT1 isoyme. Levels of message for MARCKS were down-regulated in the NMT1 knockdown cells relative to control cells. Western blot analyses of total MARCKS protein confirmed that the protein levels are reduced in the NMT1 knockdown and the double knockdown (Fig. 6). The myristoylated form of MARCKS, which migrates with an apparent molecular weight of 80 kDa, is clearly reduced in the NMT1 knockdown cells and barely visible in the double knockdown cells. MARCKS can be cleaved by calpain to produce a fragment that migrates with an apparent molecular weight of 40 kDa, and this cleavage has been shown to increase the rate of MARCKS-induced polymerization of actin (38). Levels of the p40 MARCKS product were also reduced in the NMT1 knockdown and double knockdowns.

The microarray data also indicated changes in the c-Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK/Elk signaling pathway on ablation of the NMTs. In depicted in Fig. 7, Western blot analyses showed that levels of phospho-c-Raf, phospho-MEK, and phospho—mitogen-activated protein kinase were reduced in cells treated with the NMT1-1 siRNA, whereas treatment with the NMT2-4 siRNA had no effect. In the same experiments, phosphatidylinositol 3-kinase protein and phospho-Akt levels were unaltered in any of the siRNA treatments. The maintenance of the c-Raf/MEK/ERK/Elk pathway in the NMT2-ablated cells is consistent with the proliferation data presented above. Interestingly, both c-Src activation and signaling through the c-Raf/MEK/ERK/Elk pathway have been implicated in the promotion of cellular replication (39, 40). The modulation of the c-Src activity profile in Fig. 6 is consistent

![FIGURE 6](image_url)

**FIGURE 6.** Effects of NMT siRNAs on MARCKS and c-Src. HT-29 cells were treated with 40 nmol/L NMT1-1 siRNA, 40 nmol/L NMT2-4 siRNA, 40 nmol/L each of NMT1-1 and NMT2-4 siRNA, or 80 nmol/L negative control for 48 hours as described in Materials and Methods. Western blots were prepared using 50 μg total cellular protein. The p80 signal in the MARCKS blot is the intact cellular protein, whereas the p40 band is the cleaved COOH terminus of the protein. See Table 1 for a complete list of antibodies used in these studies.

![FIGURE 7](image_url)

**FIGURE 7.** Effects of NMT siRNAs on signal transduction, cell cycle, and apoptosis proteins. HT-29 cells were treated with 40 nmol/L NMT1-1 siRNA, 40 nmol/L NMT2-4 siRNA, 40 nmol/L each of NMT1-1 and NMT2-4 siRNA, or 80 nmol/L negative control for 48 hours as described in Materials and Methods. Western blots were prepared using 50 μg total cellular protein. See Table 1 for a complete list of antibodies used in these studies.
with its activation of the c-Raf/MEK/ERK/Elk pathway in these cells.

Figure 7 shows Western blots using a panel of antibodies against a selection of proteins that regulate the cell cycle. These data indicate that the cells are not arrested in any particular stage of the cell cycle, as the signals for cyclin D1, cyclin D3, p27, CDK4, CDK6, and phospho-CDK2 were equal in all of the treatments. Fluorescence-activated cell sorting analyses of siRNA-treated cells confirmed the Western blot data in that similar cell cycle distributions were observed under all treatment conditions (data not shown). These data serve to strengthen the argument for the specificity of the siRNA knockdowns, as they are not affecting the expression of multiple “off-target” proteins.

Finally, the microarray studies suggested that levels of the BCL-2 family of proteins are differentially regulated in the NMT knockdowns. This was of interest as the BCL-2 family of proteins is involved in regulating apoptosis by controlling mitochondrial permeability and the release of cytochrome c (41). Consistent with the TdT-mediated dUTP nick end labeling data presented in Fig. 5, the antiapoptotic proteins BCL-XL and MCL-1 were down-regulated in the NMT2 and double knockdown (Fig. 7). Phosphorylation of BAD was also down-regulated in these conditions, with strong suppression occurring in the double knockdown cells. Removal of the phosphorylation signal from BAD allows it to complex with the BCL-XL protein to promote apoptosis, and this is consistent with the marked induction of apoptosis in the double knockdown cells. The levels of the proapoptotic protein BAK were reduced ~70% in all three knockdown conditions, whereas the levels of the proapoptotic protein BAX remained relatively unchanged by the siRNA treatments. As the effects of BAX are regulated by changes in its subcellular localization, it was not necessary to elevate the cellular protein levels to promote apoptosis. These data, in conjunction with the TdT-mediated dUTP nick end labeling results, indicate that NMT2 plays an important role in regulating the BCL-2 family of proteins and their ability to promote apoptosis in tumor cells.

Antitumor Activity of NMT1-1 and NMT2-4 siRNA

To determine if the ablation of either NMT1 or NMT2 had an effect on tumor progression in vivo, a syngeneic tumor model was used (42). Female BALB/c mice were injected with $1 \times 10^6$ JC (mammary adenocarcinoma) cells and palpable tumors were allowed to form. Mice were split into four groups of four mice each. Each group was treated with the designated siRNA with one-site intratumoral injection and tumor volumes were monitored (Fig. 8A). On days 1, 4, 7, and 10, group 1 was given 10 $\mu$mol/L NMT1-1 siRNA, group 2 was given 10 $\mu$mol/L NMT2-4 siRNA, group 3 was given 10 $\mu$mol/L NMT1-1 plus 10 $\mu$mol/L NMT2-4 siRNA, and group 4 was given 20 $\mu$mol/L negative control siRNA. On day 11, the mice were euthanized and the tumors were measured for a final time and then excised. As indicated in Fig. 8A, tumor growth in animals treated with both NMT1-1 and NMT2-4 siRNA was significantly lower (50% decrease by day 11) than tumor growth in animals treated with control siRNA. The reduction in tumor growth in animals treated with NMT1-1 was almost as good (42% decrease by day 11) as the double treatment. Tumor growth was decreased by only 17% in animals treated with NMT2-4 and was not statistically significantly different from that of animals treated with the negative control siRNA. The body weights of animals in all groups increased at the same rate, indicating that the siRNA administration was not overtly toxic to the mice.

To determine if the siRNA treatments were knocking down the appropriate target in vivo, reverse transcription-PCR (RT-PCR) was done on total RNA isolated from two tumors from each group. RT-PCR was used in these experiments because the antibodies used in Figs. 2, 3, 6, and 7 react efficiently with human NMT1 or human NMT2 but do not react with the murine proteins in the JC tumor cells. Figure 8B shows that message levels for NMT1 or NMT2 were substantially reduced in tumors treated with NMT1-1 or NMT2-4 siRNAs, respectively. Because the NMT message and protein levels are well coordinated (Fig. 3), it is very likely that NMT protein expression in the tumors was also significantly depleted. Overall, these results suggest that ablating NMT1 is more effective in inhibiting tumor growth than is ablation of NMT2. Additionally, this confirms the potential efficacy of small-molecule inhibitors of NMT as an anticancer therapy.

Discussion

In studies of lower eukaryotes, their single NMT gene is essential for viability (43). Mammals possess two NMT genes; however, it is not presently known if these encode redundant or specialized isozymes. Given the critical cellular proteins that NMTs affect, such as Src-related oncoproteins, it is important to discern the specific functions of these two NMT isozymes. This may be important for the generation of antineoplastic therapies that target a particular NMT isozyme. Therefore, siRNAs have been used to knockdown NMT1, NMT2, or both NMT1 and NMT2 in ovarian and colon carcinoma cells to distinguish the roles of the two isozymes.

Using siRNA-induced selective ablation of NMT1 and NMT2, we have shown that there is cross-talk between the levels of NMT1 and NMT2 expression. Specifically, when NMT1 levels were reduced, NMT2 protein and mRNA levels rose, whereas when NMT2 levels were reduced NMT1 protein and mRNA levels remained unaffected. Although the carcinoma cells normally express more NMT1 message than NMT2 message, the data in Fig. 3 suggest that cells need to maintain a threshold level of NMT expression, because NMT2 could not be fully ablated when NMT1 was depleted. This observation is consistent with expression data in normal mouse tissue showing higher levels of NMT1 in most tissues examined (28). Although it has been shown previously that NMT2 protein levels can be altered by certain xenobiotics (44), this study is the first demonstration that the expression levels of NMT2 protein are linked to NMT1 protein levels. Although it is possible that NMT2 is part of the cell’s general stress response pathway, knocking down β-actin had no effect on NMT2 protein levels (data not shown). The mechanism for coordinated expression of the NMT isozymes is presently unknown, so it will be interesting to examine the NMT2 promoter region to determine if there are uncharacterized elements that mediate this coordinated regulation.
These studies have also shown that NMT1 and NMT2 have both unique and common substrates in cells. A preliminary examination of the abilities of NMT1 and NMT2 to modify a small set of substrate peptides indicated that they may have different specificities (26). The siRNAs described herein provide the tools to examine this issue in intact cells and have revealed an interesting dichotomy regarding the Src family of proteins. The N-myr-palm-GFP reporter used in these studies contains the NH2-terminal 14–amino acid residues of Fyn (MGCVQCKTKLTEER) fused to GFP. After removal of the methionine residue, it is myristoylated and palmitoylated, allowing its stable association with the plasma membrane (45). Thus, if Fyn is a unique substrate of either NMT1 or NMT2, it was expected that siRNAs targeting these isozymes should reveal the modifying enzyme. However, the experiments clearly showed that individually reducing the expression of NMT1 or NMT2 had no effect on the membrane localization of this modified GFP. In contrast, simultaneously reducing the levels of both NMT1 and NMT2 resulted in redistribution of the Fyn-GFP to the cytosol without affecting the plasma membrane localization of the GFP-farn-palm protein that reaches the membrane in a myristoylation-independent manner. These results suggest that both NMT isozymes are able to modify the Fyn myristoylation motif.

**FIGURE 8.** Antitumor activity of NMT siRNAs. A. Female BALB/c mice received subcutaneous injections of 10^6 JC cells suspended in PBS. NMT1-1 (■), NMT2-4 (▲), NMT1-1 and NMT2-4 (●), or negative control (▲) siRNAs were injected intratumorally on days 1, 4, 7, and 10, and tumor volumes were measured. Inset, body weights for the animals during this study. *, P < 0.05, unpaired t tests. B. RT-PCR analyses of NMT mRNA levels in siRNA-treated tumors. RT-PCR analyses were performed using 1 μg total RNA isolated from the indicated tumors and primers described in Materials and Methods. NMT1 message was amplified 22, 23, or 25 cycles, whereas NMT2 was amplified 23, 24, or 26 cycles.
This finding is significant because it has been proposed that the specificities of these isozymes might result from differential subcellular localization through the divergent NH₂ termini (46).

In contrast to the finding with the Fyn myristoylation signal, c-Src, which is myristoylated but not palmitoylated, seems to be primarily processed by NMT1. Although there is not yet evidence for a direct interaction between NMT1 and c-Src, the present data consistently indicate that c-Src is not myristoylated in cells that lack NMT1 and therefore cannot localize to the plasma membrane where it is activated. The increased protein levels of c-Src in the NMT1 knockdown cells may reflect an attempt to compensate for decreased c-Src activity. Further, these results show that loss of c-Src phosphorylation leads to the loss of FAK phosphorylation, and this is consistent with the requirement of c-Src to be myristoylated before its interaction with FAK at the plasma membrane. These results are also consistent with the finding that NMTs are overexpressed in colon cancer, which could underlie the increased Src activity observed in these and other tumors. It is possible that the increase in NMT1 protein levels drives all of the available c-Src to the plasma membrane, thereby increasing the local concentration of c-Src where it is required to activate downstream effectors.

The differential processing of Fyn and c-Src could identify sequence differences that make one a substrate for both NMT enzymes and the other specific for NMT1. This may reflect processing selectivity for proteins that are only myristoylated versus those that are further processed by palmitoylation. Further characterization of a more diverse set of GFP constructs, as well as examination of the myristoylation of endogenous cellular proteins, in the NMT knockdowns will be necessary to fully explain this issue.

Consistent with NMT1 modification of c-Src, data presented in this study indicate that NMT1, but not NMT2, is involved in cellular proliferation, because DNA synthesis was decreased with the NMT1-1 and double siRNA treatment but not with the NMT2-4 siRNA treatment. The Western blot data suggest that a critical NMT1 substrate is c-Src, which requires myristoylation for membrane association and its ability to transform cells (7, 47). Further, Src activation is involved in several aspects of cellular transformation, including proliferation, survival, migration, and invasion (48-50). Additionally, activity of the Src-family of kinases is required for signal transducers and activators of transcription 3 and FAK phosphorylation (51), and this phosphorylation is required for the progression of the cell cycle (52, 53). Furthermore, it has been reported that overexpression of FAK leads to an accelerated G₁-S–phase transition, whereas overexpression of a dominant-negative FAK inhibits cell cycle progression at G₁ (39, 40). These authors also reported that the dominant-negative FAK competed for binding to c-Src, which resulted in a decrease in ERK (mitogen-activated protein kinase) activation. These results are consistent with our findings that ablation of NMT1 leads to loss of c-Src and FAK activation as well as down-regulation of the c-Raf/MEK/ERK/Elk pathway. Zhao et al. have shown that disrupting FAK activation and down-regulating the c-Raf/MEK/ERK/Elk pathway leads to a slowing of cellular replication (54). The data presented above clearly show that replication is slowed when NMT1 activity is inhibited. Overall, these findings suggest that loss of NMT1 is responsible for slowing of the replication process due to the decreased activation of c-Src.

In contrast to the cellular replication, cell survival seems to be regulated by both NMT1 and NMT2. Consequently, simultaneous ablation of NMT1 and NMT2 was particularly lethal to the cells, producing 29-fold increase in the apoptosis index. This is consistent with experiments with fungi (55), Arabidopsis thaliana (56), and flies (57) that have shown that loss of all NMT activity is lethal to these organisms. Additionally, compounds that inhibit both NMT1 and NMT2 are cytotoxic to tumor cells (31). Western blot analyses of the BCL-2 family of apoptosis regulators showed that there is a shift toward induction of apoptosis when NMT2 is ablated from the cells. At the present time, there is no clear target protein for NMT2 that can be linked to this process. It is of interest to note that SK-OV-3 cells are p53 null and that HT-29 cells have elevated levels of p53, indicating that the effects of loss of both NMT1 and NMT2 are p53 independent and therefore may be useful for inducing apoptosis in a wide range of human tumors. Using the tools established in this study, it is now possible to explore the substrate specificity of the individual NMT isozymes to determine which protein(s) NMT2 regulates to affect this pathway.

Finally, in vivo studies consisting of intratumoral injection of siRNAs against the NMT isozymes indicate that pharmacologically reducing the activity of NMT1 will have antitumor activity. Although clearly not optimized in terms of delivery and schedule, the siRNAs provide preliminary proof-of-principle that inhibitors of NMT1 will be useful as therapeutic agents. This is consistent with the antitumor activity of small-molecule inhibitors of NMT that we have recently described (31). Interestingly, the data indicate that knocking down NMT2, which had the greatest effect on apoptotic induction in vitro, had little effect on tumor progression in vivo. This was not due to lack of in vivo ablation of NMT2, because the NMT1-1 and NMT2-4 siRNAs caused pronounced reductions in the message levels for NMT1 and NMT2, respectively. The rate of in vivo tumor growth reflects the relative rates of cell proliferation and apoptosis. The JC cells growing as xenografts represent an aggressive tumor model, with an in vivo doubling time of ~3 days (Fig. 8). Under these conditions, the rate of tumor growth is dominated by the rate of proliferation because the apoptotic index is very low. Therefore, it is not surprising that depletion of NMT1, which is more closely related to proliferation than is NMT2, has a stronger growth suppressive effect than does depletion of NMT2. It is also possible that the tumors may develop mechanisms to evade the deleterious effects of loss of NMT2. In either case, definition of selective substrates for NMT1 and NMT2 will be important for understanding the mechanisms of the differential antitumor effects of the siRNAs.

Because NMT activity is essential for the growth and survival of a variety of infectious organisms, NMT has been considered as a target for the development of new antibiotics (55, 58-60). For example, several studies have targeted the NMTs of Candida albicans and Cryptococcus neoformans, which are responsible for systemic fungal infections in
immunocompromised humans, as treatment of such infections (43, 61-64). NMTs have also been targeted for the development of antiviral drugs due to their essential role in viral replication. For example, the myristoylated glycinic residues of several viral Gag proteins have been mutated, and this prevents the assembly of viral particles (65-67). We can now add antineoplastic therapies to the growing list of possible uses for NMT inhibitors. The results presented in this study provide evidence that targeting a specific NMT isozyme could have dramatic effects on tumor cells reliant on these enzymes for survival. It is possible to envision NMT1-specific inhibitors affecting the growth of c-Src-driven colon tumors while having little effect on nonmetastatic tissues in the body.

In conclusion, we have described siRNA sequences that efficiently and selectively ablate the expression of the two NMT isozymes in human cells. These tools have allowed the functional characterization of human NMT1 and NMT2, providing insight into the cellular processes involving these isozymes. The knockdown system will also aid in the development of specific inhibitors for NMT1 and NMT2, which could provide a much needed therapy for diseases, such as colon and ovarian cancer.

**Materials and Methods**

**Cell Culture**

SK-OV-3 cells (ATCC-HTB-77) were maintained in DMEM containing 10% fetal bovine serum, 50 μg/mL gentamicin, and 1 mmol/L sodium pyruvate at 37°C in an atmosphere of 5% CO2 and 95% air. HT-29 cells (ATCC-HTB-38) were maintained in RPMI containing 10% fetal bovine serum with 50 μg/mL gentamicin at 37°C in an atmosphere of 5% CO2 and 95% air. DMEM and RPMI, fetal bovine serum, gentamicin, and sodium pyruvate were from Life Technologies (Gaithersburg, MD).

** Constructs**

**siRNA Constructs.** Oligonucleotide templates for the generation of siRNAs were designed using the Ambion siRNA Target Finder (http://www.ambion.com). Four sets of oligonucleotides were designed for each gene encoding NMT1 (National Center for Biotechnology Information accession no. NM_021079) and NMT2 (National Center for Biotechnology Information accession no. NM_004808), and the siRNAs were synthesized using the Silencer siRNA construction kit (Ambion). The siRNA used to knockdown NMT1, designated NMT1-1, is 5'-AAATGAGGGGACAACAGCTAC-3' and was determined to be optimally effective at 10 nmol/L. The siRNA used to knockdown NMT2, designated NMT2-4, is 5'-AAAAAGTTTGACTAGTACTAC-3' and was also determined to be optimally effective at 10 nmol/L. Transfections using both NMT1 and NMT2 siRNAs were done at a final total concentration of 20 nmol/L. Negative control siRNA templates were obtained from Ambion (Negative Control 1 siRNA Templates) and used to generate the negative control siRNA, which was used at a concentration of 20 nmol/L in all experiments.

**GFP Constructs.** The GFP-farn-palm construct (also designated as pEGFP-F) was purchased from Clontech (Mountain View, CA) and contains the COOH terminus of H-Ras fused to GFP. The N-my-palm-GFP construct was generated by the addition of the NH2-terminal myristoylation signal of Fyn to the NH2 terminus of GFP by PCR. The following primers were used in the PCR reaction: 5'-GGGAATTCACTAGGCGTGTTG-CAATGTAAAGACAAACTGACGGAGGGTGAG-CAAGGGGAGGACGTG-3' and 5'-GGGGATCCCTATCA-GGACGTTCACCAGCTGACGGAGGAGAGGGTGAG-CAAGGGGAGGACGTG-3'. The PCR fragment was digested with EcoRI and BamHI restriction enzymes and cloned into the pZEOsv-2 expression vector (Invitrogen, Carlsbad, CA). Once clones were generated, the construct was isolated from bacteria and sequenced to ensure its identity. This construct was used in transient transfection assays at 0.66 μg per reaction.

**Transfection with siRNAs**

Transfection of cells with siRNA was done as follows: cells were plated in six-well or 10 cm dishes in either RPMI or DMEM with 10% fetal bovine serum (without antibiotic) such that cells were 40% to 50% confluent at the time of transfection. Transient transfection of siRNA constructs into both cell types was achieved using 7.5 μg/mL LipofectAMINE 2000 (Invitrogen) for 4 hours at 37°C. Following the 4-hour incubation, the medium was replaced with RPMI or DMEM plus 10% fetal bovine serum (without antibiotic) and the cells were incubated at 37°C for 24 to 72 hours. Cells were harvested at the indicated times, and each sample was divided into two aliquots. One cell aliquot was used for Western blot analysis and the other for Northern blot analysis using standard methods as described below.

**Western Blot Analyses**

Following the transfection, harvested cells were resuspended in hypotonic lysis buffer [10 mmol/L HEPES (pH 7.4), 10 mmol/L KCl, 1.5 mmol/L MgCl2, and 5 μmol/L phenyl-methylsulfonyl fluoride] containing 1% SDS. The protein concentration in each sample was determined using the fluorescein assay (68), and samples normalized for equal amounts of protein (50-100 μg/lane) were separated on 10% SDS-PAGE gels and electrophoresed to polyvinylidene difluoride membranes. The membranes were incubated in PBST [100 mmol/L sodium phosphate, 100 mmol/L NaCl (pH 7.6) containing 0.1% Tween 20] or TBST [20 mmol/L Tris, 137 mmol/L NaCl (pH 7.6) containing 0.1% Tween 20] plus 5% (v/v) powdered milk at room temperature for 2 hours. For the phosphospecific blots, the membranes were washed thrice in TBST at room temperature for 5 minutes. Membranes were then incubated with various antibodies (Table 1) in TBST (phosphospecific antibodies) plus 5% bovine serum albumin or PBST on a shaking platform for 18 hours at 4°C. The membranes were washed thrice in TBST (phosphospecific antibodies) or PBST (10 minutes each at room temperature), and anti-mouse antibodies conjugated to horseradish peroxidase (Sigma, St. Louis, MO) were then incubated with the membranes in TBST (phosphospecific antibodies) or PBST containing 3% powdered milk (w/v) on a shaking platform for 1 hour at room temperature. The blots were washed four times in TBST (phosphospecific antibodies) or PBST for 5 minutes each at room temperature. Blots were developed with SuperSignal development reagents (Pierce, Rockford, IL). Filters were stripped of the original antibody by incubation in stripping buffer containing 100 mmol/L 2-mercaptoethanol, 2% SDS, 0.1% SDS, and 0.1% Triton X-100 on a shaking platform for 18 hours at 4°C. Blots were then incubated for 2 hours at room temperature in TBST (phosphospecific antibodies) or PBST. The membranes were washed thrice in TBST (phosphospecific antibodies) or PBST for 5 minutes each at room temperature. Blots were then incubated with the membranes in TBST (phosphospecific antibodies) or PBST containing 3% powdered milk (w/v) on a shaking platform for 1 hour at room temperature. The blots were washed four times in TBST (phosphospecific antibodies) or PBST for 5 minutes each at room temperature. Blots were developed with the SuperSignal reagents (Pierce, Rockford, IL). Filters were stripped of the original antibody by incubation in stripping buffer containing 100 mmol/L 2-mercaptoethanol, 2% SDS, 0.1% SDS, and 0.1% Triton X-100 on a shaking platform for 18 hours at 4°C. Blots were then incubated for 2 hours at room temperature in TBST (phosphospecific antibodies) or PBST. The membranes were washed thrice in TBST (phosphospecific antibodies) or PBST for 5 minutes each at room temperature. Blots were then incubated with the membranes in TBST (phosphospecific antibodies) or PBST containing 3% powdered milk (w/v) on a shaking platform for 1 hour at room temperature. The blots were washed four times in TBST (phosphospecific antibodies) or PBST for 5 minutes each at room temperature. Blots were developed with the SuperSignal reagents (Pierce, Rockford, IL). Filters were stripped of the original antibody by incubation in stripping buffer containing 100 mmol/L 2-mercaptoethanol, 2% SDS,
and 62.5 mmol/L Tris-HCl (pH 6.7) for 30 minutes at 50°C and were then probed with antibodies against pan-actin. Blots were imaged with a Fujifilm (Bedford, United Kingdom) Intelligent Dark Box II, and the appropriate bands were quantified using Image Gauge 4.0 software. More information on the imager can be found at http://home.fujifilm.com/products/science/si_product/las1000plus/spc.html.

**Northern Blot Analyses**

After transfection, total RNA was isolated from the harvested cell aliquots using the RNasey total RNA isolation kit (Qiagen, Valencia, CA). Total RNA (~ 10 µg) was loaded onto 1% agarose gels containing formaldehyde, electrophoresed for 2 hours at 70 V, and transferred to a nylon membrane (Immobilon NY+, Millipore, Billerica, MA) and UV cross-linked. The membrane was sequentially hybridized with the NMT1 cDNA (1,492 bp), a 500-bp PCR fragment of the NMT2 gene (~ 1 to +503), and a β-actin probe (1,076 bp) obtained from Ambion. All probes were random prime labeled with [α-32P]dCTP to a specific activity of 10⁸ cpm/µg. The membranes were exposed to film, and the densities of appropriate bands were quantified using ImageQuant software.

**Subcellular Localization of GFP**

Transfections of SK-OV-3 cells for GFP localization analyses were carried out as described above with the following modification. The cells were plated into 24-well culture dishes containing sterile glass coverslips and transfected with siRNAs as indicated above, except that 0.66 µg vector encoding either N-myr-palm-GFP or GFP-farn-palm was included. At 48 hours after transfection, the cells were fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature. Coverslips were mounted on glass slides with mounting medium (Sigma) and sealed with rubber cement. The cells were visualized by epifluorescence microscopy and images were captured digitally.

**Cell Proliferation Assay**

SK-OV-3 or HT-29 cells were transfected in six-well plates as described above and incubated at 37°C in an atmosphere of 5% CO₂ and 95% air for 48 hours. At 48 hours after transfection, [³H]thymidine (16 µCi) was added to each well and cultures were incubated for an additional 2 hours. The medium was then removed by aspiration and 0.8 mL ice-cold 10% trichloracetic acid was added to each well. After 10 minutes, the trichloracetic acid was removed by aspiration and replaced with 0.4 mL of 40 µg/mL type I DNA (Sigma) in 0.2 mol/L NaOH. The samples were incubated at 37°C for 30 minutes and scraped and the amount of [³H] in the supernatant containing the recovered genomic DNA was quantified by scintillation counting.

**Apoptosis Assay**

HT-29 or SK-OV-3 cells were plated on glass coverslips and transfected with siRNAs as indicated above. After 48 hours, the cells were permeabilized and cells with fragmented DNA were labeled following the instructions for the In situ Cell Death Detection kit, Fluorescine (Roche, Indianapolis, IN). Fluorescent and bright-field images were captured digitally and the percentage of cells undergoing apoptosis was calculated as the ratio of fluorescent cells to total cells.

**NMT siRNA Tumor Study**

Female BALB/c mice (6-8 weeks old) were injected subcutaneously in the right hind flank with 1×10⁶ JC (mammary adenocarcinoma) cells suspended in PBS (42). On the appearance of palpable tumors, tumor volumes were determined using calipers measuring the length (L) and width (W) of the tumor and calculated using the formula: (L × W²) / 2. Groups of four mice were treated with single-site intratumoral injections of NMT1-1 (10 µmol/L), NMT2-4 (10 µmol/L), NMT1-1 and NMT2-4 (20 µmol/L total), or negative control (20 µmol/L) siRNA in a total volume of 50 µL PBS on days 1, 4,
7, and 10. Animals were euthanized and tumors were excised and weighed on day 11. Whole body weight and tumor measurements were done on days, 1, 4, 7, 10, and 11. Ps were determined by unpaired t tests using GraphPad InStat (San Diego, CA).

RT-PCR Analysis

Total RNA was isolated from flash-frozen tumors using the RNasey RNA isolation kit (Qiagen). Total RNA (1 μg) was used in each RT-PCR reaction using the One-Step RT-PCR kit (Qiagen). NMT1 mRNA was amplified using primers that span the intron between exons 10 and 12. The 5′ primer is 5′-GCAACATGGAACAAAGC-3′ and the 3′ primer is 5′-GGTACGTGTTATTTGACGACCAG-3′. The NMT1 primer pair generated a 487-bp PCR product. NMT2 mRNA was amplified using primers that span the intron between exons 10 and 12. The 5′ primer is 5′-CAGGTTTGAGACCAATGGAAACC-3′ and the 3′ primer is 5′-TCTTTTTGGAATGG-CAGTTCC-3′. The NMT2 primer pair generated a 566-bp PCR product. RT-PCR products were resolved on a 2% agarose gel.

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References

phosphorylation and vascular endothelial growth factor signaling, signal transducer and activator of transcription 3 and focal adhesion kinase.


