Expression of MTAP Inhibits Tumor-Related Phenotypes in HT1080 Cells via a Mechanism Unrelated to Its Enzymatic Function

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ABSTRACT Methylthioadenosine Phosphorylase (MTAP) is a tumor suppressor gene that is frequently deleted in human cancers and encodes an enzyme responsible for the catabolism of the polyamine byproduct 5′-deoxy-5′-methylthioadenosine (MTA). To elucidate the mechanism by which MTAP inhibits tumor formation, we have reintroduced MTAP into MTAP-deleted HT1080 fibrosarcoma cells. Expression of MTAP resulted in a variety of phenotypes, including decreased colony formation in soft-agar, decreased migration, decreased in vitro invasion, increased matrix metalloproteinase production, and reduced ability to form tumors in severe combined immunodeficiency mice. Microarray analysis showed that MTAP affected the expression of genes involved in a variety of processes, including cell adhesion, extracellular matrix interaction, and cell signaling. Treatment of MTAP-expressing cells with a potent inhibitor of MTAP’s enzymatic activity (MT-DADMe-ImmA) did not result in a MTAP− phenotype. This finding suggests that MTAP’s tumor suppressor function is not the same as its known enzymatic function. To confirm this, we introduced a catalytically inactive version of MTAP, D220A, into HT1080 cells and found that this mutant was fully capable of reversing the soft agar colony formation, migration, and matrix metalloproteinase phenotypes. Our results show that MTAP affects cellular phenotypes in HT1080 cells in a manner that is independent of its known enzymatic activity.

Methylthioadenosine phosphorylase (MTAP) is a widely expressed metabolic enzyme in the methionine salvage pathway that converts the polyamine byproduct 5′-deoxy-5′-methylthioadenosine (MTA) into adenine and methylthioribose-1-phosphate (Kamataki and Carson 1981; Olopade et al. 1995). Loss of either MTAP protein or the MTAP gene is frequent in a large number of different human tumors, including leukemias, lymphomas, mesothelioma, lung carcinoma, pancreatic carcinoma, squamous cell carcinoma, biliary tract cancer, glioblastoma, osteosarcoma, and neuroendocrine tumors (Stadler and Olopade 1996; Dreyling et al. 1998; Hori et al. 1998; Schmid et al. 1998; Wong et al. 1998; Brat et al. 1999; M’soka et al. 2000; Garcia-Castellano et al. 2002; Illei et al. 2003; Chen et al. 2004; Subhi et al. 2004; Hustinx et al. 2005; Karikari et al. 2005). The most frequent mechanism for MTAP inactivation is homozygous deletion of the 9p21 region, where both MTAP and the CDKN2A/ARF tumor suppressor gene complex are located (Nobori et al. 1996). Because these deletions generally inactivate CDKN2A/ARF as well as MTAP, it was initially hypothesized that loss of MTAP in tumors was simply due to it being a coincident bystander. However, there is now substantial evidence that MTAP itself has tumor suppressor activity. Re-expression of MTAP in MTAP deleted MCF-7 breast cells results in loss of anchorage-independent growth in vitro and loss of tumor formation in vivo (Christopher et al. 2002). In addition, re-expression of MTAP in either a MTAP-deleted melanoma cell line or a gastric carcinoma cell line causes reduced cellular invasion in vitro (Behrmann et al. 2003; Kim et al. 2011). Mice heterozygous for a germline deletion of MTAP die prematurely of T-cell lymphoma and have accelerated B-cell lymphoma onset when crossed to Eμ-myc mice (Kadariya et al. 2009, 2013). Finally, germline mutations in humans that disrupt primate specific MTAP exons are associated with diaphyseal medullary stenosis with malignant fibrous histiocytoma,

KEYWORDS cell mobility and migration suppressor genes methionine

Copyright © 2015 Tang et al.
doi: 10.1534/g3.114.014555
Manuscript received September 23, 2014; accepted for publication November 5, 2014; published Early Online November 11, 2014.
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Supporting information is available online at http://www.g3journal.org/lookup/suppl/doi:10.1534/g3.114.014555/-/DC1
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a rare genetic disease associated with bone dysplasia and cancer (Camacho-Vanegas et al. 2012).

The mechanism by which MTAP affects tumorigenesis is not understood. Previously, it was shown that MTAP expression caused decreased ornithine decarboxylase (ODC) levels and reduced polyamine levels in both yeast and mammalian cells (Subhi et al. 2003; Chattopadhyay et al. 2005; Tang et al. 2006). Because elevated polyamines and ODC are common in cancer cells, it has been speculated that this might be important for MTAP’s tumor suppressor function (Subhi et al. 2004). A second possible mechanism relates to MTAP’s substrate, MTA. Data from yeast and mammalian cells indicate that loss of MTAP results in large elevations of MTA (Kamatani and Carson 1980; Chattopadhyay et al. 2006; Stevens et al. 2009). Because MTA is a competitive inhibitor of methyltransferase enzymes, including histone and DNA methyltransferases (Williams-Ashman et al. 1982), it is possible that loss of MTAP may have effects on the epigenetic control of gene expression in tumor cells.

Although the loss of MTAP is associated with tumorigenesis, pharmacologic inhibition of MTAP can have antitumor activity. Singh et al. have developed a transition-state inhibitor of MTAP, MT-DADMe-ImmA, that binds with extremely high affinity (86 nM Kd) and completely abolishes enzyme activity (Singh et al. 2004). Using this inhibitor, Basu et al. (2007) demonstrated that the growth of a MTAP+ human head and neck cancer cell line could be inhibited in a xenograft mouse model. Although superficially this seems to contradict the idea that loss of MTAP promotes tumorigenesis, it is important to remember that the drug may be exerting its antitumor effects not on the tumor directly but indirectly via its effects on stromal cells. In addition, this antitumor effect was only shown to occur in MTAP+ cells not MTAP− cells.

In the experiments described here, we have characterized the phenotype of an MTAP-deleted HT1080 human fibrosarcoma cell line in which we have stably reintroduced the MTAP gene. Our results show that MTAP expression inhibits several tumor-related phenotypes and causes global changes in gene expression, affecting several cellular pathways controlling cell adhesion and signaling. However, treatment of these MTAP-expressing cells with the MTAP inhibitor MT-DADMe-ImmA, or expression of a mutated version of MTAP, did not reverse these effects. Our findings suggest that MTAP suppresses tumorigenicity in HT1080 cells via a function that is unrelated to its known enzymatic activity.

**MATERIALS AND METHODS**

**MTAP-expressing cell lines**

MTAP− (M−), MTAP+ (M+), and D220A cells were created by stably transfecting either the pTRE2-MTAP, pTRE2-MTAP:D220A, or pTRE2 empty plasmid into HT1080 cells (containing a homozygous MTAP deletion) and pooling 10 individual expressing clones together as was previously described (Tang et al. 2012). HT1080 cells (Clontech Laboratories, Mountain View, CA) were cultured in Dulbecco’s modified Eagle medium (DMEM) medium supplemented with 2 mM glutamine, 100 µg/mL penicillin, 100 µg/mL streptomycin, 10% fetal bovine serum, and 250 µg/mL G418. Clones were selected using 250 µg/mL hygromycin from a 50 mg/mL stock solution in phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO). MT-DADMe-ImmA was used at a concentration of 10 µM for all experiments and was obtained from Dr. Vern Schramm (Albert Einstein Medical Center, Bronx, NY). MTA, putrescine, and 2-difluoromethyl-ornithine (DFMO) were obtained from Sigma Aldrich. All media, serum, and antibiotics were obtained from the tissue culture facility at Fox Chase Cancer Center.

**MTAP and ODC activity assay**

Protein extracts were prepared from cells lysed in M-PER mammalian protein extraction reagent (Pierce, Rockford, IL) with 1× Complete Mini protease inhibitor (Roche Biochemical, Indianapolis, IN) or tissue homogenized by using a dounce homogenizer in PBS containing 10% glycerol with the aforementioned protease inhibitor. The extracts were centrifuged at 10,000 × g for 15 min at 4°, and the supernatants were collected. Protein concentration was measured with BCA kit (Pierce Rockford, IL). MTAP activity was determined with a photometric assay to measure adenine production as described previously (Christopher et al. 2002). ODC activity was assayed by measuring the 14CO2 formed by decarboxylation of 14C-labeled L-ornithine in 30 min at 37° as described previously (Subhi et al. 2003). 14C-labeled L-ornithine with specificity 5 mCi/mmol was purchased from Moravek Biochemicals (Brea, CA). One unit of MTAP catalyzes the formation of 1 µmol of adenine/mg/minute, whereas 1 unit of ODC catalyzes the formation of 1 nmol of CO2/mg/hr.

**Soft agar growth and cell invasion assay**

Cells were assessed for growth in soft agar as previously described (Christopher et al. 2002). Cells were tested for invasive ability using BD BioCoat Matrigel Invasion chambers (Becton Dickinson, Bedford, MA). Five hundred microliters of media containing 1 × 104 cells was added to each well and incubated for 24 hr at 37° in 5% CO2. Cells were fixed and stained with methanol and stained Giemsa. Noninvading cells on the upper surface of the filter were removed by wiping out with a cotton swab, and the filter was excised and mounted on a microscope slide. Invasiveness was quantified by counting cells on the lower surface of the filter.

**Wound healing assay**

Cells were inoculated at 2 × 105 per well in 6-well plate and grown to near confluence. They were then scratched with a sterile10-µL pipette tip, rinsed briefly with medium to remove unadhered cells, and reincubated in medium. Wound closure was inspected and photographed at 0, 8, and 24 hr with Nikon Eclipse TS100 microscope and Nikon DXM1200 Digital Camera (Melville, NY).

**Xenograft studies**

The M+ and M− cells were transfected with pZsGreen1-N1 vector (Clontech) that expressed ZsGreen1 protein. The green fluorescent ZsGreen1 protein excited at 493 nm and emitted at 505 nm. Because our MTAP+ and MTAP− cells already expressed neomycin resistance (the marker of the ZsGreen1 vector), a linear puromycin marker (Clontech) was used and cotransfected in a ratio 20:1 (vector: linear marker). Cells were cultured in DMEM medium supplemented with 2 mM glutamine, 100 µg/mL penicillin, 100 µg/mL streptomycin, 10% fetal bovine serum, 250 µg/mL G418, and 1 µg/mL puromycin. Nine individual, puromycin-resistant, high-fluorescent M+ and M− clones were pooled to make the M+ + GFP (M+G) and M− + GFP (M−G) cell lines.

Severe combined immunodeficiency (SCID) mice were subcutaneously injected with 6 × 106 M+G or M−G cells in 200 µL of DMEM. Tumor cell growth was monitored by fluorescent imaging with an IVIS Spectrum Imager (PerkinElmer, Waltham, MA). After 6 wk, mice were killed and tumors were dissected. After the tumor mass was recorded, the tumor was homogenized in PBS with 10% glycerol.
and 1 x Complete Mini proteinase inhibitor (Roche, Indianapolis, IN) using a dounce homogenizer and MTAP activity was measured.

**Polyamine measurements**

Intracellular polyamines were measured on a Biochrom 30 amino acid analyzer using a sodium citrate buffer and a polyamine ion exchange column as previously described (Christopher et al. 2002).

**DNA microarray analysis**

M+ and M− cells were grown in 100 mM plates until 80% confluent, harvested, and total RNA was extracted using RNeasy Mini Kit (QIAGEN, Valencia, CA) according to manufacturer’s instruction. The quantity and quality of the RNA was assessed with the Agilent 2100 Bioanalyzer. Ten micrograms of total RNA was used to create cDNA by using the One-Cycle cDNA Synthesis kit (Affymetrix, Santa Clara, CA). Biotin-labeled cRNA synthesis was performed using the Affymetrix In Vitro Transcription Labeling Kit. The biotinylated cRNA samples were cleaned up, fragmented, and then hybridized to human GeneChip (Human Genome U133 plus 2.0; Affymetrix) in an Affymetrix GeneChip Hybridization Oven 640 according to manufacturer’s protocols. The washing procedures were carried out automatically in Affymetrix GeneChip Fluidics Station 450. The processed GeneChip was scanned with Affymetrix GeneChip Scanner 3000 7G. Highly expressed genes were those determined to have a signal of 50 or greater.

**Statistical analysis**

Affymetrix microarray data were analyzed using methods implemented in the R/Bioconductor platform. Raw expression data in the form of Affymetrix CEL files were preprocessed using the Robust Multi-chip Average (RMA) method (Irizarry et al. 2003). RMA combines three pre-processing steps: background correction, between-array quantile normalization, and summarization of 25-mer probe intensities into probe set intensity measures.

**Quantitative real-time polymerase chain reaction (RT-PCR)**

Gene-specific probes and primer sets for quantitative Taqman Assays were obtained from Applied Biosystems (Foster, CA). Human β-actin was used as an endogenous normalization for the expression of genes of interest. Quantitative RT-PCR was carried out according to the TaqMan Assay-on-Demand one-step protocol of Applied Biosystems under universal thermal condition in triplicate with ABI-Prism 7900 HT Real Time PCR system.

**Zymography**

Extracellular matrix-degrading matrix metalloproteinases (MMPs) present in the cells were subjected to electrophoresis on precast 10% gelatin-containing polyacrylamide gels (Invitrogen, Carlsbad, CA), and their activities detected as a transparent band against a blue background. In brief, cell lysates (25 μg/lane) were mixed with 2X Tris-Glycine

![Figure 1](image-url) MTAP expression and activity in HT1080 cells. (A) Western blot showing levels of MTAP in extracts from stably transfected HT1080 cells. M− is the parent cell line transfected with vector alone (pTRE2). M+ has been transfected with the MTAP expressing construct pTRE2:MTAP. M+I is the identical to M+, except the cells have been treated for 72 hr with 10 μM the MTAP inhibitor, MT-DAD-Me-ImmA. Hela contains extract from a MTAP+ Hela cell. D220A contains extract from a HT1080 cell that has been transfected with a plasmid that expresses D220A MTAP (pTRE2:MTAP:D220A). (B) MTAP enzymatic activity measured in the same extracts as used in (A). Error bars show SD of enzyme assay (n = 4). All means are different from each other as assessed by one-way ANOVA followed by Tukey test (P < 0.01 for all comparisons).
SDS sample buffer from Invitrogen (no heating and no addition of reduce reagent), loaded on gels and electrophoresed in 1X Tris-Glycine SDS Running Buffer (Invitrogen) at 125 V and room temperature for about 90 min. After electrophoresis, gels were washed twice with PBS containing 2.5% Triton X-100 for 5 min/each, incubated in 1X Zymogram Renaturing Buffer (Invitrogen) for 45 min, and equilibrated in 1X Zymogram Developing Buffer (Invitrogen) for 35 min at ambient temperature with gentle agitation. The equilibrated gels were incubated in refreshed Zymogram Developing Buffer at 37°C overnight or longer for maximum sensitivity and optimal results and stained with SimplyBlue SafeStain (Invitrogen) according manufacturer’s instruction.

**Western blotting**

Western blotting was performed as previously described (Christopher et al. 2002). Polyclonal rabbit MTAP antibody (Cell Signaling Technology, Danvers, MA) was used at 1:1000 dilution. Monoclonal mouse α-Tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:2000 dilution. After washing, the membranes were incubated with secondary antibody conjugated to horseradish peroxidase at 1:30000 dilution. Membranes were developed using enhanced chemiluminescent reagent (Pierce, Rockford, IL).

**MTA quantitation**

Extracts were prepared by sulfocyclic acid extraction as previously described (Gupta et al. 2009). To remove excess salt, 100 μL of extract was subjected to high-performance liquid chromatography (HPLC) purification using an Agilent 1100 HPLC system (Agilent, Wilmington, DE) containing a Xterra MS C18 column (Waters, Milford, MA) and eluted with 25% MeOH and 75% H2O.

For LC/MS/MS analysis, a Waters 2690 Alliance HPLC instrument and an LCQ Classic ion trap mass spectrometer (Thermo, San Jose, CA) were used. The purified sample (10 μL) was injected into a Vydak TP C18 column (GRACE, Deerfield, IL). The analysis was carried out in isocratic mode, 30% MeOH, 70% H2O, and 0.1% formic acid with a flow rate 50 μL/min. The experimental conditions for LCQ were as follows: the spray voltage was set at 5 KV, the sheath gas flow rate was set at 60 (arb), the capillary voltage was set in a range of 20—40 V. Quantitation of MTA was performed by comparing peak area to an internal [5’—2H3] MTA standard that was synthesized by the

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**Figure 2** Functional effects of MTAP expression in HT1080 cells. (A) Growth of indicated cell lines in soft agar. Ten thousand cells were plated in each well and image was taken after 14 d. Labeling is identical to Figure 1. (B) Invasion of cells through matri-gel. Indicated cells are expressed as a percentage of the M- line. All assays performed in nine separate wells (n = 9). SD is indicated by bars. Different letters above each bar indicates that the means of all columns are different from each other as assessed by one-way ANOVA followed by Tukey test (P < 0.01 for all comparisons). (C) MTAP expression and wound healing. Confluent monolayers of the indicated cells were scratched and wound healing was monitored at 0, 8, and 24 hr as described in the Materials and Methods. Results are expressed as percent of wound openness. Error bars show SEM (n = 6). (D) Cell extracts (50 μg) from the indicated strains were loaded onto 10% gelatin-containing polyacrylamide gels and stained as described in the Materials and Methods. White bands indicate where MMPs have digested gelatin.
in-house organic synthesis facility. The molecular weight of \([5^t-\text{H}_2]\) MTA is three units greater than standard MTA. The quantitative measurement of \([5^t-\text{H}_2]\) MTA and MTA from the cell lysates and cell media samples was performed by setting the LCQ ion trap mass spectrometer in the selected reaction monitoring scan mode to achieve maximum sensitivity. For MTA, transitions m/z 298.00 → 136.10 and m/z 298.00 → 162.80 were monitored. For \([5^t-\text{H}_2]\), transitions m/z 301.10 → 136.10 and m/z 301.10 → 165.90 were monitored. All measurements, including samples for making a calibration curve, triplicate samples from cell lysate, and triplicate samples from cell media, were performed in the same day to avoid potential variance.

RESULTS

MTAP acts as a tumor suppressor for HT1080 cells

HT1080 cells are an immortalized human fibrosarcoma cell line that lacks MTAP expression (Tang et al. 2000). We created MTAP+ (M+) and MTAP− (M−) HT1080 cell lines by stably transfecting either a MTAP expression plasmid or an empty vector control and pooled multiple clones together to minimize the effects of integration events (Tang et al. 2012). The amount of MTAP protein and activity in M+ cells were slightly reduced from those observed in Hela cells (Figure 1, A and B; compare lane M+ with Hela), indicating that MTAP is being expressed in these cells at near physiologic levels. (We will discuss lanes labeled M+1 and D220A in the last two sections.) We performed several assays to examine the effects of MTAP expression on tumor-related phenotypes. Under standard tissue culture conditions, we observed no difference in doubling time between M+ and M− cells (25.6 ± 0.8 vs. 24.8 ± 0.8 hr, \(P = \text{ns}\)) and no obvious differences in overall cellular morphology (Supporting Information, Figure S1). However, we did observe a dramatic decrease in the ability of M+ cells to form colonies on soft agar (Figure 2A). In addition, we found that the M+ cells exhibited a 55% reduction (\(P < 1 \times 10^{-6}\)) in migration through a basement membrane preparation (Matrigel) using a Boyden chamber assay compared with M− cells (Figure 2B). We also found that M− cells showed increased mobility in a wound-healing assay (Figure 2C).

Because MMPs have been shown to be important for invasion through matrigel, we examined metalloprotease activity. This was done using gel zymography, a technique in which gelatin is embedded in the gel and metalloprotease activity is detected by digestion of the gelatin. These studies found that MTAP expression greatly inhibited both MMP-9 and MMP-2 activity (Figure 2D). Quantitative RT-PCR of MMP-9 message indicates that this down-regulation is occurring at the level of mRNA (Figure S2). These findings show that expression of MTAP can inhibit cellular functions related to migration and invasion.

Finally, we injected M+ and M− cells expressing a ZsGreen1 protein tag (see the section Materials and Methods) into SCID mice to determine whether MTAP expression could affect tumor cell growth in a mouse xenograft model. Four of the five mice injected with M+ cells had reduced tumor burden after 4 wk compared with mice injected with M− cells as judged by the level of ZsGreen1 protein fluorescence (Figure 3A). In addition, the mean weight of the excised tumor was reduced by 73% (Figure 3B). To confirm the MTAP status of the cells, we also measured MTAP activity in the excised tumors (Figure 3C). We found that the tumors excised from mice injected with M− cells had a 70% reduction in MTAP activity, indicating that the majority of cells in the tumor were derived from the injected M− cells (Figure 3C). These findings show that MTAP expression inhibits the tumor formation of HT1080 cells in mice.

MTAP expression alters HT1080 gene expression profile

To identify the molecular pathways responsible for MTAP’s antitumorigenic phenotypes, we performed RNA microarray profiling using Affymetrix arrays. Using a criteria of at least a twofold difference in expression and a false discovery rate <0.01, we identified 283 probe sets that were up-regulated and 64 probe sets that were down-regulated by MTAP, between M+ and M− cells (Table S1). As there were 17,475 probe sets expressed above background on the array, this means that 2% of the probes were at least twofold differentially regulated. This list of 347 probes identified a total of 254 unique transcripts, which were then examined to see whether they were enriched in any particular functional pathways. Using the KEGG pathway database, we found enrichment for genes in several pathways involved in cell adhesion, cell communication, and cell migration (Table S2). The
pathways identified are consistent with our functional observations that loss of MTAP affects colony formation in soft-agar, cell migration, and expression of MMPs. Interestingly, several genes specific to the Wnt-signaling pathway were identified. This pathway has been shown to control processes involved in cell migration, adhesion, and differentiation (Neth et al. 2007; Guo et al. 2008).

To confirm the microarray results, quantitative RT-PCR was used to examine the RNA levels of eight of the genes identified as having altered regulation by MTAP. These genes were selected because they were identified as being involved in Wnt or other signaling pathways (Figure 4). Six of the selected genes were found to be up-regulated, and two were down-regulated on the microarray. We found that seven of the eight genes tested confirmed the findings of the microarray analysis.

MTAP expression does not affect polyamine or ODC activity in HT1080 cells

Previously, our group reported that expression of MTAP in MCF-7 breast adenocarcinoma cells also repressed soft agar growth and tumor formation in mice. In these cells, MTAP expression resulted in decreased ODC activity and decreased levels of intracellular polyamines (Christopher et al. 2002). Because ODC is a known oncoprotein (Germer and Meyskens 2004), these findings suggested a possible mechanism for MTAP's tumor suppressor effects. To see whether this also occurred in HT1080 cells, we measured intracellular polyamine levels and ODC activity in the M+ and M− cells (Figure S3). Unlike MCF-7 cells, we did not observe any significant differences in either polyamine or ODC activity. In addition, although soft-agar colony formation in MCF-7 cells was inhibited by DFMO (an irreversible inhibitor of ODC activity) and stimulated by putrescine (the product of the ODC reaction), we failed to see any such effects in HT1080 cells (Figure S4). These results show that in HT1080 cells, MTAP expression does not exert its phenotypic effects by modulation of ODC or polyamine levels.

MTA accumulation does not explain MTAP’s phenotypic effects

An alternative hypothesis to explain the effects of MTAP expression on cellular phenotypes involves the accumulation of MTAP’s substrate, MTA. MTA is known to inhibit a variety of methyltransferase enzymes including histone and DNA methyltransferases (Williams-Ashman et al. 1982), and exogenous MTA added to either melanoma or hepatocellular carcinoma cell lines can alter expression of MMP and growth factor genes (Stevens et al. 2009; Kirovski et al. 2011). Therefore, it seemed possible that this might be the mechanism by which MTAP could influence the RNA expression levels of a large number of genes. To test this idea, we treated M+ cells with MT-DAD-Me-ImmA, a drug that is a transition state analog and is a potent inhibitor of MTAP enzymatic activity (Singh et al. 2004). To confirm that the compound was actually inhibiting MTAP function, we measured enzyme activity in MT-DAD-Me-ImmA−treated cells (M+, Figure 1B) and found that drug treatment resulted in a 93% reduction in enzyme activity. We also examined both intra- and extracellular MTA levels using LC-MS/MS in M−, M+, and M+I cells. As
expected. M+ cells had very low levels of both intracellular and extracellular MTA compared with M− cells, showing that the presence of MTAP dramatically affects MTA levels (Figure 5A). In M+I cells, we found that both intracellular and extracellular MTA levels were actually slightly greater than those observed in M− cells. These findings indicate that the MT-DAD-Me-ImmA is effectively inhibiting MTAP activity and causing significant MTA accumulation.

We next compared the gene expression profiles in all three cell lines (M−, M+, and M+I). Our expectation was that M+ cells treated with the inhibitor would show gene expression profiles similar to M− cells. Thus, we expected that a comparison of M+I vs. M+ differentially expressed genes would have substantial overlap with the M− vs. M+ list. However, this was not the case. Only one (2%) of the M− vs. M+ up regulated genes were found in the M+I vs. M+ up-regulated gene set, and only three (5%) of the down-regulated genes were in common (Figure 6A). In contrast, the gene expression pattern in M+I cells was much more similar to M+ cells, with 79% of the up-regulated genes and 50% of the down-regulated genes being in common. To explore this further, we compared the mean fold-difference in gene expression in all 347 probe sets that were identified as either up or down regulated in M+ cells relative to M− to the mean fold difference observed in the M− vs. M+I comparison (Figure 6B). For induced genes, the mean log2 fold change was 1.45 in M− vs. M+ compared with 1.52 in M− vs. M+I. For repressed genes, the mean log2 fold change was 1.37 in M− vs. M+ compared with 1.03 in M− vs. M+I. These results show that the addition of the inhibitor did not greatly affect the ability of MTAP to affect the mRNA levels of downstream genes. We also observed the similar effects on the quantitative PCR of the selected differentially regulated signaling genes shown in Figure 4. These results indicate that majority of the downstream transcriptional effects of MTAP occur even when its enzymatic activity is inhibited.

To control for any nonspecific effects of the inhibitor, we also performed a separate array experiment in which we compared M− cells and M− cells plus inhibitor. We did not observe any genes that were differentially expressed using the same selection criteria (twofold difference, false discovery rate < 0.01). These results show that the inhibitor is quite specific for MTAP, and that the differences observed between M+ and M+I cells are due to MTAP inhibition.

Finally, we examined the effect of MT-DAD-Me-ImmA on several different functional assays related to tumor formation. MT-DAD-Me-ImmA treatment of M+ cells failed to promote growth in soft-agar (Figure 2A) and did not reverse the inhibitory effect of MTAP on wound healing (Figure 2C). However, MT-DAD-Me-ImmA treatment did cause a slight increase in MMP-9 and MMP2 levels (Figure 2D) and some increase in invasion potential (Figure 2B).

**Mutant MTAP expression has similar effects as wild type MTAP in HT1080 cells**

Aspartate 220 is a critical residue in the MTAP catalytic site that is necessary for enzymatic activity. Previously, our laboratory had shown that D220A MTAP failed to suppress soft-agar colony formation and elevated polyamine levels in MTAP-deleted MCF-7 cells (Christopher et al. 2002). Therefore, we decided to test the effects of this mutation in HT1080 cells. As described in the Methods section, we created a novel HT1080 cell line, D220A that stably expresses D220A MTAP. Western analysis shows that the mutant protein is abundantly expressed, but has less than 3% of the enzyme activity present in cells expressing wild-type MTAP (Figure 1). These cells were then examined using three functional assays: growth in soft agar, wound healing, and MMP-2 and MMP-9 activity (Figure 2A, A and C). In all of these assays, D220A cells behaved identically to M+ cells. These findings support the view that MTAP’s tumor suppressor function in HT1080 cells does not require its known enzyme activity.

**DISCUSSION**

In the experiments described here, we have assessed the consequences of ectopic MTAP expression on an MTAP deleted human fibrosarcoma—derived cell line. MTAP expression suppressed the ability of these cells to grow in SCID mice, their ability to grow on soft agar, and their ability to invade through a collagen matrix. All of these phenotypes support the idea that MTAP is a tumor suppressor gene and are consistent with our previous study that MTAP acted as a tumor suppressor gene in a MCF-7 breast cancer cell line (Christopher et al. 2002).

At the molecular level, we found that MTAP expression in HT1080 cells resulted in the significant alteration of the gene expression profile. Specifically, MTAP affected the expression of genes involved in a number of important cellular functions including cell adhesion, cell communication, and cell migration. Our findings are consistent with reports that MTAP expression in gastric carcinoma cells inhibits migration in a wound-healing assay, and that inhibition of MTAP causes increased expression of MMP-1 and MMP-9 in a human hepatocellular carcinoma cell line (Kim et al. 2011). Given the effects of MTAP expression on invasion, migration, and soft agar growth, it was particularly interesting that MTAP affected the expression of several genes involved in the Wnt-signaling pathway. The Wnt-signaling pathway frequently is found activated in a variety of human cancers and is thought to play a key role in a variety of developmental processes related to cell proliferation.
migration and epithelial to mesenchyme transition (Neth et al. 2007). In our studies, several of the Wnt-signaling transcripts were elevated when MTAP was expressed. However, it is unclear whether this reflects increased Wnt-signaling or a response to lower Wnt-signaling. Further studies will be needed to clarify this point.

The key finding of this study was that MTAP protein, but not enzymatic activity, was required for MTAP's tumor suppressor effects on HT1080 cells. Inhibition of MTAP's enzymatic activity with a potent transition state inhibitor failed to significantly reverse any of the functional effects of MTAP expression, despite effectively inhibiting MTAP's enzyme activity in cell extracts and causing the accumulation of MTA. This finding is not consistent with the idea that MTA inhibition of histone and DNA methyltransferases are behind MTAP's effect on tumorigenesis. In addition, expression of a catalytically inactive mutant form of the enzyme (D220A) was able to suppress soft-agar colony formation and MMP-2 and MMP-9 expression. This particular result was unexpected given our previous finding that this same mutant was not effective in suppressing tumor formation in MCF-7 cells (Christopher et al. 2002), and the findings of Kirovski and Stevens that exogenous MTA can up-regulate growth factors and MMPs in melanoma and hepatocellular carcinoma cell lines (Kirovski et al. 2011; Stevens et al. 2009). However, there are some important differences between these experiments and the ones reported here. In MCF-7 cells, MTAP expression reduces the steady-state levels of polyamines and causes a significant decrease in ODC activity, whereas in HT1080 cells, neither of these effects is observed. Furthermore, in MCF-7 cells soft-agar colony formation is inhibited by DFMO and stimulated by putrescine, but this also was not observed in HT1080 cells. With regards to the data reported by Bosserhoff et al., it is important to note that although there is clearly an effect of exogenous MTA on gene expression and various functional assays, the effects are relatively modest, and it is unclear whether these effects alone are sufficient to explain all of the biological effects of MTAP-loss. Taken in total, these findings suggest that MTAP may exert its tumor suppressor effects via two different mechanisms; an enzyme-dependent mechanism that may involve the accumulation of the MTAP substrate MTA, and a nonenzymatic mechanism that predominates in HT1080 cells.

What are the possible nonenzymatic functions of MTAP? Global proteomic studies carried out in MTAP+ Hela cells have identified 20 proteins that appear to form complexes with MTAP (Table S3). These include proteins that are involved in a variety of different molecular functions including vesicle trafficking, purine metabolism, transcription/chromatin regulation, cytoskeletal function, and RNA metabolism. Interestingly, the ortholog of MTAP in Saccharomyces cerevisiae, MEU1, was initially identified in a mutant screen specifically designed to identify genes involved in transcriptional regulation (Donoviel and Young 1996). These findings are all consistent with the idea that MTAP may have a nonenzymatic function that may involve interactions with other proteins. Future studies will need to focus on the elucidation of these functions.

In summary, the studies described here show that MTAP acts as a tumor suppressor gene in HT1080 cells, affecting functions related to cell adhesion, cell communication, and cell invasion. MTAP expression causes a large change in the cells gene expression profile, but this effect is not dependent on MTAP's known enzymatic function. Our
results show that MTAP has additional nonenzymatic functions that play an important role in its tumor suppressor function.

ACKNOWLEDGMENTS

We acknowledge the contribution of the Genomics Facility and Yue-Sheng Li (Fox Chase Cancer Center) for technical assistance. We thank Vern Schramm (Albert Einstein College of Medicine) for supplying the MT-DAD-Me-ImmA. This work was supported by National Institutes of Health core grant CA06927, National Cancer Institute grants CA131024 (W.K.), an appropriation from the Commonwealth of Pennsylvania, and the PA CURE fund.

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Communicating editor: R. Sclafani