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Tumor metastasis-associated human *MTA1* gene and its MTA1 protein product: Role in epithelial cancer cell invasion, proliferation and nuclear regulation

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Abstract

Using differential cDNA library screening techniques based on metastatic and nonmetastatic rat mammary adenocarcinoma cell lines, we previously cloned and sequenced the metastasis-associated gene *mta1*. Using homology to the rat *mta1* gene, we cloned the human *MTA1* gene and found it to be over-expressed in a variety of human cell lines (breast, ovarian, lung, gastric and colorectal cancer but not melanoma or sarcoma) and cancerous tissues (breast, esophageal, colorectal, gastric and pancreatic cancer). We found a close similarity between the human *MTA1* and rat *mta1* genes (88% and 96% identities of the nucleotide and predicted amino acid sequences, respectively). Both genes encode novel proteins that contain a proline rich region (SH3-binding motif), a putative zinc finger motif, a leucine zipper motif and 5 copies of the SPXX motif found in gene regulatory proteins. Using Southern blot analysis the *MTA1* gene was highly conserved, and using Northern blot analysis *MTA1* transcripts were found in virtually all human cell lines (melanoma, breast, cervix and ovarian carcinoma cells and normal breast epithelial cells). However, the expression level of the *MTA1* gene in normal breast epithelial cells was $\sim$ 50% of that found in rapidly growing adenocarcinoma and atypical epithelial cell lines. Experimental inhibition of MTA1 protein expression using antisense phosphorothioate oligonucleotides resulted in inhibition of growth and invasion of human MDA-MB-231 breast cancer cells with relatively high MTA1 expression. Furthermore, the MTA1 protein was localized in the nuclei of cells transfected with a mammalian expression vector containing a full-length *MTA1* gene. Although some MTA1 protein was found in the cytoplasm, the vast majority of MTA1 protein was localized in the nucleus. Examination of recombinate MTA1 and related MTA2 proteins suggests that MTA1 protein is a histone deacetylase. It also appears to behave like a GATA-element transcription factor, since transfection of a GATA-element reporter into *MTA1*-expressing cells resulted in 10–20-fold increase in reporter expression over poorly *MTA1*-expressing cells. Since it was reported that nucleosome remodeling histone deacetylase complex (NuRD complex) involved in chromatin remodeling contains MTA1 protein and a MTA1-related protein (MTA2), we examined NuRD complexes for the presence of MTA1 protein and found an association of this protein with histone deacetylase. The results suggest that the MTA1 protein may serve multiple functions in cellular signaling, chromosome remodeling and transcription processes that are important in the progression, invasion and growth of metastatic epithelial cells.

Introduction

Several genes have been identified as metastasis-associated genes, and at least some of these genes are associated with progression or metastasis of carcinoma cells [1, 2]. Examples are: *mst1, nm23, WDNM1, WDNM2, pGM21, stromelysin-3, KAI-1, BRMS1, KiSS1* and *MKK4* genes [3–10]. Although for the most part direct evidence for the roles of these specific genes and their encoded products in particular steps of the metastatic process is not yet known for certain, these genes are generally over-expressed or under-expressed in metastatic cells compared to their nonmetastatic counterparts and thus they are considered candidates for being metastasis-associated genes.

Our efforts in this area started when we cloned a novel candidate metastasis-associated gene, *mta1* [11, 12], which was isolated by differential cDNA library screening using the 13762NF rat mammary adenocarcinoma metastatic system [13]. We found that *mta1* mRNA was differentially expressed in highly metastatic rat mammary adenocarcinoma cell lines [11–13]; however, the function of the *mta1*

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gene product was unknown. We have now cloned the human homologue *MTA1* gene, characterized this gene and investigated the putative function of its encoded product [14, 15].

MTA1 protein has been localized in the cell nucleus, and it is thought that its major function is associated with its nuclear location [14, 15]. Recently, two groups reported that nucleosome remodeling histone deacetylase complex (NuRD complex), which is involved in chromatin remodeling, contains MTA1 protein or a MTA1-related protein (MTA2) [16, 17]. Thus, a possible function for the MTA1 protein has been reported; however, the exact role of the MTA1 protein in tumor progression and metastasis must still be determined. Here, we will discuss the structure and possible function of the *MTA1* gene and its encoded MTA1 protein product.

MTA1 gene and protein sequence analysis

The nucleotide sequences of both the rat *mta1* and human *MTA1* genes have been determined [11, 12, 14]. The human *MTA1* gene (accession number U35113) was found to be 88% identical to the rat *mta1* sequence, and the human *MTA1* gene encoded a putative protein of 715 amino acid residues with a predicted molecular weight of $\sim$82 kDa. The amino acid sequences of the rat and human proteins were 96% identical and 98% similar (Figure 1) [11, 14]. Similar to the rat Mta1 protein, the human MTA1 protein contained a proline-rich stretch (LPPRPPPPAP) at the carboxy-terminal end of the molecule at residues 696-705. This sequence completely matched the consensus sequence for the src homology 3 domain-binding site, XPXXPPPFXP [18] or XpFPpXP [19] (where X stands for nonconserved residues, P for proline, p for residues that tend to proline, and F for hydrophobic residues) (Figure 2) [15].

Due to increased interest in transcription we examined whether MTA1 protein was a possible DNA-binding or nuclear transcription factor. In this analysis of the human MTA1 protein, we also found a putative zinc finger DNA binding motif Cys-X2-Cys-X17-Cys-X2-Cys [20] beginning at residues 393, and a leucine zipper motif [21] beginning at residue 251. These sequences were also conserved in the rat Mta1 protein (Figure 1). The human MTA1 protein was rich in SPXX motifs, and these are known to occur frequently in gene regulatory and DNA-binding proteins [22]. The human MTA1 protein contained five SPXX sequences (Figure 1), corresponding to frequencies of 7.09×10^{-3} which is $\sim$2.5 times the average protein frequency (2.89×10^{-3}). Furthermore, the MTA1 protein encoded three putative nuclear localization sequences (using the PSORT prediction software) (Figure 1) [11, 12, 14]. We have also found a SANT domain in MTA1, and this type of domain was recently reported to be similar to the DNA-binding domain of myb-related proteins [23]. A SANT domain has been identified in SWT3, a yeast component of the SWI/SNF complex [24], along with ADA2, a component of the histone deacetylase complex [25], N-CoR, a nuclear hormone co-repressor [26], and TFIIIB subunit B,

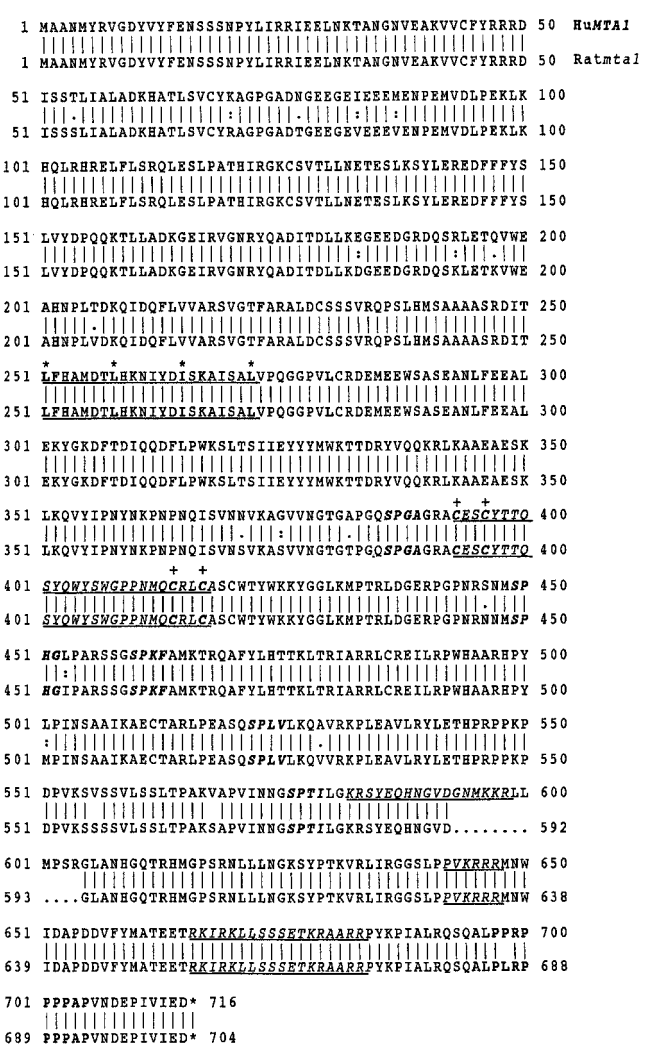

Figure 1. The predicted amino acid sequences of the human MTA1 and rat mta1 proteins. The identical amino acid residues (96%) between human MTA1 and rat Mta1 proteins are indicated by (|), well-conserved replacements by (:) and less conserved (.) [11, 12]. The underlined polypeptide sequences 251–273 are characteristic of a lucine zipper motif. The underlined and italic polypeptide sequences 393–417 are characteristic of a GATA-type zinc finger motif. Five SPXX motifs are also present and conserved in both human MTA1 and rat Mta1 proteins. Three and two putative nuclear localization sequences (shown in underlined and italic figures) are in human MTA1 and rat Mta1, respectively. The C-terminal proline rich region found previously starts at amino acid residue 696 of the human MTA1 protein (from [14] with permission).

SH3-Binding Motifs in Some Common Proteins

Gene	*Sequence No.*	*SH3-Binding Motifs*											
Consensis 1		X	P	X	X	P	P	P	Φ	X	P		
Consensis 2		-	-	-	X	P	Φ	P	P	X	P		
Rat Mta1	684-695	L	P	L	R	P	P	P	P	A	P	V	N
Human MTA1	787-797	L	P	L	R	P	P	P	P	A	P	V	N
Murine 3BP1	267-277		P	T	M	P	P	P	L	P	P	V	P
Murine 3BP2	2-12		P	A	Y	P	P	P	P	V	P	V	P
Murine SOS1	1146-1156		E	V	P	V	P	P	P	V	P	P	P
Murine SOS1	1174-1184		H	L	D	S	P	P	A	I	P	P	R
Murine SOS1	1285-1295		H	S	I	A	G	P	P	V	P	P	R
Murine Formin	873-883		P	P	T	P	P	P	L	P	P	P	L
Rat m4 mAchR	277-286		P	A	L	P	P	P	V	R	P	V	P
Human PI3K p85	91-101		P	P	R	P	L	P	P	A	P	G	S
Human PI3K p85	303-313		P	A	P	A	L	P	V	K	P	P	K
Human GAP CDC42	250-260		A	P	K	P	M	P	P	R	P	P	L
Human HK2	93-103		G	V	R	P	L	P	P	L	P	D	P
Human proacrosin	333-343		P	P	R	P	L	P	P	R	P	P	A
Human dynamin	785-795		A	P	A	V	P	P	A	R	P	G	S
Human dynamin	811-821		G	A	P	P	V	P	S	R	P	G	A
v-Fgr	12-22		R	P	R	P	L	P	P	L	P	P	T

Figure 2. Comparison of the Src homology 3 domain-binding site in rat Mta1, human MTA1 and various other proteins (from [15] with permission).

a basal pol III transcription factor in yeast [27]. The SANT domain has also been referred to as the MFY domain since it has many aromatic amino acid residues [16]. There were also two highly acidic regions located in the 200 MTA1 amino-terminal residues. These highly negatively charged regions are characteristic of the acidic activation domains of many transcription factors [28].

We next examined whether the MTA1 gene was highly conserved. To assess the extent of evolutionary conservation of the *MTA1* gene we analyzed genomic DNA of several species by Southern blot analysis. Strong genomic signals were detected in monkey and yeast, moderate signals in human, rat, mouse, dog, cow and rabbit, and weak signals were detected in chicken [11]. Thus, the *MTA1* gene was conserved in all species examined, and it is likely a highly conserved protein in evolution.

MTA1 gene expression in various cell lines

The rat *mta1* gene was isolated using a differential expression procedure and rat mammary adenocarcinoma cell lines. We found that the rat *mta1* gene was over-expressed in highly metastatic rat mammary carcinoma cells compared to poorly metastatic or nonmetastatic rat mammary cells [11–13]. To determine the expression of the human *MTA1* gene in non-tumorigenic and tumorigenic cells, we examined 14 cell lines of human origin. Transcripts for the *MTA1* gene were found in virtually all cell lines analyzed [15]. Interestingly, human breast cancer MDA-MB-231 cells of high metastatic potential strongly expressed the *MTA1* gene, whereas MDA-MB-435 cells of poor metastatic potential [29] expressed the *MTA1* gene at very low levels [11, 12]. The expression level of the *MTA1* gene in a normal breast epithelial cell line (Hs578Bst) with slow growth rate was from one-third to one-half that seen in breast adenocarcinoma cells and atypical breast cells (HBL-100) with a rapid growth rate [15]. The relative expression (normalized with respect to GADPH expression) of the *MTA1* gene in various human cell lines from highest to lowest was as follows: MDA-MB-231, HeLa > SKOV-3, ZR-75-1, HBL-100, A2058 > OVCA-433, OVCA-432, Ovcar-3, HT-29, KM-12C, Hs578Bst > MBA-MD-435, OVCA-429 [15]. Thus, the *MTA1* gene was expressed at various levels among different cell lines. Although the expression of the *MTA1* gene in animal and human cell lines generally followed metastatic potential, there were some exceptions to this that may reflect differences in the cells or alterations during cell culture [15].

MTA1 gene expression in human tumors

To examine the expression of the *MTA1* gene in human tissues we obtained tumor biopsy specimens from various epithelial cancers. We initially focused our attention on breast cancers because preliminary results suggested over-expression of the *MTA1* gene in malignant breast carcinomas compared to surrounding normal tissues. The majority of 20 invasive breast carcinomas over-expressed *MTA1* gene (tumor/normal ratio > 2) compared to surrounding normal tissue. Similarly, *MTA1* gene was over-expressed in malignant gastric and esophageal carcinomas [30, 31]. In 14/36 colorectal carcinomas and 13/34 gastric carcinomas the *MTA1* gene was over-expressed (tumor/normal ratio > 2). Tumors that over-expressed *MTA1* RNA showed significantly higher rates of invasion and lymph node metastasis and tended to have higher rates of vascular involvement.

MTA1 protein localization in breast cancer cells

We examined the distribution of the MTA1 protein inside cells using microscopy. Using indirect immunofluorescence we found that the MTA1 protein accumulated in the nucleus of breast cancer cells [14]. This nuclear immunoreactivity was present in many large, intense foci that were not detected near the nuclear membrane. However, using the anti-MTA1 reagent the nucleolus region was negative for fluorescence [14].

MTA1 gene and invasion and growth

Two important properties of malignant cells are their abilities to invade surrounding tissues and to grow at near and distant sites from the primary tumor. To directly demonstrate a role for the *MTA1* gene in breast cancer cell invasion and growth we used the technique of antisense inhibition of *MTA1* gene expression. Although this procedure is not without its limitations [32], we employed phosphorothioate oligonucleotides (PONs) as antisense oligodeoxynucleotides with prolonged lifetime [33, 34]. Human breast cancer MDA-MB-231 and MDA-MB-435 cells were treated with PONs for 4 h, after which their proliferation was monitored for several days [35]. Antisense PONs markedly inhibited the cell growth of MDA-MB-231 cells to 22% of mock-treated cells and to 28% of sense PONs treated cells at 72 h, respectively (Figure 3A). In contrast, antisense PONs did not affect the growth of the nonmetastatic MDA-MB-435 cells (Figure 3B). We also examined the ability of the PONs to inhibit invasion in an *in vitro* invasion assay [36]. Antisense PONs inhibited cell invasion of MDA-MB-231 cells compared to sense PONs treated cells at 48 and 72 h (Figure 3C).

Using immortalized keratinocytes and squamous carcinoma cells and sense and antisense *MTA1*, Mahoney et al. [37] recently found that *MTA1* gene expression is associated with migration and invasion and is necessary but not sufficient for anchorage-dependent survival mediated by a Bcl-x(L) anti-apoptotic mechanism. They also found that MTA1 expression in immortalized keratinocytes depends, at least in part, on signaling through the epidermal growth factor receptor [37]. Thus, the expression of the *MTA1* gene is associated with several important steps in the metastatic process.

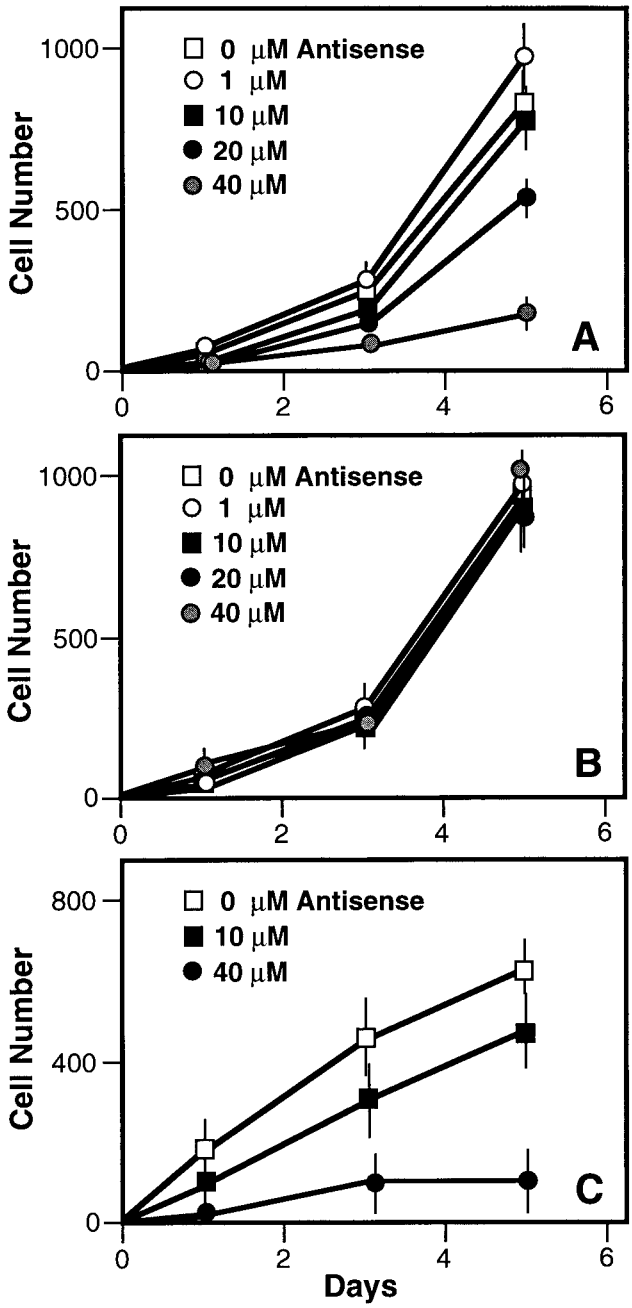

Figure 3. Effect of MTA1 antisense PONs on the growth and invasion of MDA-MB-231 (A) or MDA-MB-435 (B) cells. Time-course of cell growth inhibition using MTA1 antisense PONs were evaluated as described in [15]. Only the MDA-MB-231 cells were growth inhibited by the MTA1 antisense PONs (from [15] with permission). MDA-MB-231 cells were also treated with antisense or sense PONs to assess inhibition of invasion in an *in vitro* invasion assay (C). Only the antisense PONs inhibited invasion.

MTA1 antisense oligonucleotides decrease MTA1 protein levels

Antisense PONs are useful in determining the function of targeted proteins, but first it is necessary to confirm that they indeed suppress protein levels in recipient cells. To confirm that the inhibition of cell invasion and proliferation by antisense PONs was involved in the suppression of the MTA1 protein amounts in cells, the MTA1 protein levels of MDA-MB-231 cells treated with antisense PONs and

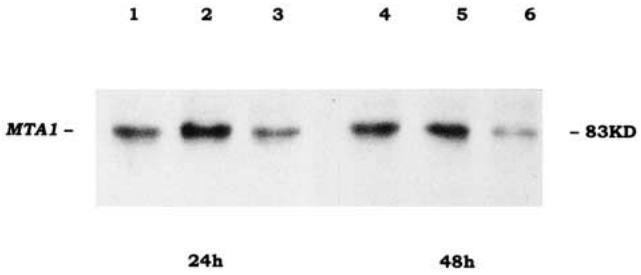

Figure 4. Effect of MTA1 antisense PONs on the expression of the MTA1 gene in MDA-MB-231 cells. Cells were cultured for 24 (lanes 1–3) or 48 (lanes 4–6) hours after transfected MTA1 antisense PONs as described in Materials and Methods. The cells were harvested, lysed, proteins separated by SDS-PAGE, and the gel was immunoblotted with anti-human MTA1 antibody as described in 'Materials and methods'. Ten microgram of cell protein extract was loaded for each sample. Lanes 1 and 4, no PONs; lanes 2 and 5, MTA1 sense PON; lanes 3 and 6, MTA1 antisense PON. In this figure, the level of MTA1 protein in cells treated with MTA1 antisense decreased to 30% of the level without PON or using MTA1 sense PON at 48 h (from [15] with permission).

sense control were examined by Western blot analysis using anti-MTA1 protein polyclonal antibodies. The anti-MTA1 protein recognized a 83 kDa protein band that was identified as identical in migration to the MTA1 protein. This band disappeared after preincubation of anti-MTA1 protein with the immunogen oligopeptides [14, 15]. To verify that the antisense effects were due to inhibition of target gene expression we quantitated MTA1 protein expression in MDA-MB-231 cells treated with antisense PONs or sense control PONs. Antisense PONs inhibition of the *MTA1* gene resulted in an approximately 70% reduction of MTA1 protein levels as detected by Western blot analysis in the antisense treated cells after two days, whereas in cells treated with sense control PONs there was no effect on the MTA1 protein levels (Figure 4).

MTA1 protein and histone deacetylase complex

The MTA1 protein sequence suggested that it may be functional in the nucleus, and it also was localized in the nucleus by immunomicroscopy. Therefore, we sought to determine if it interacted with nuclear proteins. Interestingly, a nucleosome remodeling histone deacetylase complex (NuRD complex) involved in chromatin remodeling, contains the MTA1 protein or a MTA1-related protein (MTA2) [16, 17]. Using a double-labeling procedure we found that the MTA1 protein is physically associated with histone deacetylase 1 (HDAC1) in a protein complex (NuRD complex) [38].

The MTA1 protein and tumor invasion and metastasis

From the information above we concluded that the metastasis-associated *MTA1* gene is a novel, highly conserved gene that encodes a nuclear protein product, one that could be involved in chromosome modifications or alterations. The human MTA1 protein also appears to be well conserved with only a 4% divergence at the amino acid sequence level between the human and rat genes [11, 14, 15].

The putative functional domains like the SH3-binding motif [18, 19], GATA-type zinc finger motif [20, 39], leucine zipper motif [21] and the SPXX motifs [22] were highly conserved between the predicted human and rat protein sequences. The *MTA1* gene was expressed in all tumor cell lines analyzed thus far, but similar to the rat *mta1* gene we found different quantities of *MTA1* transcripts in various cells. With the exception of the human breast cancer cell line MDA-MB-435, we found that the expression level of the *MTA1* gene in untransformed breast epithelial cells was 28–50% of that found in breast cancer or atypical mammary cell lines. In general, the more progressed mammary cells with higher amounts of MTA1 protein grow at faster rates, suggesting that the *MTA1* gene might be involved in the process of cellular proliferation. Epithelial cancers that over-expressed *MTA1* RNA showed significantly higher rates of invasion and lymph node metastasis and tended to have higher rates of vascular involvement. In addition, the *MTA1* gene was found to be over-expressed in invasive and metastatic squamous carcinoma cells where it contributes to migration, invasion and loss of apoptosis [37].

The MTA1 protein is likely involved in important functions in normal cells. We previously found that the rat *mta1* gene was expressed at low levels in normal tissues, with the exception of the testis [11]. In the testis spermatogenesis occurs as a highly controlled and complex process typified by a high rate of cell proliferation that is tightly regulated by a number of growth factors and cytokines. Thus the MTA1 protein might be involved in normal cellular functions, such as cell proliferation.

Variants of the *MTA1* gene have been isolated by Kumar et al. [39] as short forms of *MTA1* gene that contain a unique sequence. One of these smaller forms of MTA1 protein contains an estrogen receptor (ER)-binding motif and may act as a co-repressor of nuclear ERα. This short form may act by sequestering ER in the cytoplasm and enhancing its non-genomic response to ER. Deleting the ER-binding motif in the short form of MTA1 restores its nuclear localization and abolishes its co-repressor function and ER-interactions. Thus, the short form of MTA1 may be important for redirecting nuclear receptor signaling by preventing ER from entering the nucleus [39].

To determine if the *MTA1* gene is involved in the regulation of tumor cell invasion and proliferation we [14, 15] and others [37] used antisense oligonucleotide treatment of breast cancer cell lines that show different levels of expression of the *MTA1* gene. Antisense PONs against the *MTA1* gene inhibited the cell growth and *in vitro* invasion of MDA-MB-231 breast cancer cells. Specific inhibition of gene expression by the use of antisense PONs has been used extensively but these procedures are not without their problems [32–34]. We found that after transfection of antisense PONs MTA1 protein decreased to 20–30% of that found in sense-treated cells within 2 days, but we failed to find significant changes in the amounts of MTA1 protein with the sense sequence PONs, indicating that the growth and *in vitro* invasion inhibition by antisense PONs was a sequence-specific effect. Also, we could not demonstrate an effect of antisense PONs on the growth inhibition of cells with a low level of MTA1 protein expression. The mechanism of cell growth and survival regulation by MTA1 protein is not known, but it is thought to be controlled, in part, by signaling through growth factor receptors at the cell surface or in the cytoplasm [1].

The MTA1 protein may be used in a complex whose function is to modify or remodel chromosomes. When the *MTA1* gene was transfected and expressed in 293Tcells, the MTA1 protein localized within the nuclear matrix. A nucleosome remodeling histone deacetylase complex (NuRD complex) involved in chromatin remodeling contains the MTA1 protein or a MTA1-related protein (MTA2) [16, 17]. Trichostatin A, a potent specific inhibitor of histone deacetylase (HDAC), causes G1/G2 arrest in fibroblasts [40]. Moreover, HDAC1 which is a component of the NuRD complex has been shown to interact with Rb to repress transcription [41–43]. Acetylation of the C-terminal of p53 modified its ability to bind to DNA [44]. Therefore, the MTA1 protein might interact with or may even be a part of the histone deacetylase and could act as a co-activator of this complex. In support of this notion, MTA1 has a unique protein primary structure that suggests that it might function in signal transduction [1] and DNA-binding [38]. The MTA1 protein is the first mammalian protein found that contains the motif Cys-X2-Cys-X17-Cys-X2-Cys, which is a zinc-finger domain that also appears in GATA transcription factors. The same zinc-finger domain configuration has been found in *GLN3, areA* and *nit-2*, major regulatory factors for nitrogen metabolism in *Saccharomyces cerevisiae, Aspergillus* and *Neurospora*, respectively [45, 46]. The *nit-2* protein recognizes an identical core sequence of TATCTA, and a recent study has also shown that the GLN3 protein binds the nitrogen upstream activation sequence of *GLN1*, the gene that encodes glutamine synthetase [45]. Thus it is plausible that the MTA1 protein binds to a specific sequence of DNA and regulates gene expression.

The MTA1 protein is likely a nuclear regulatory protein, and it might interact with specific genes involved in cellular regulation. In preliminary experiments we have found that the expression of the *MTA1* gene was increased four-times in *c-erbB2/neu* stable transfectants of MDA-MB-435 cells compared to untransfected cells. The MTA1 protein is also associated with histone deacetylase 1 (HDAC1) in a NuRD protein complex [38]. Determination of the role of the MTA1 protein in nuclear protein complexes will be necessary to confirm our notion that the *MTA1* gene is involved in gene and cell growth regulation, invasion and the progression of epithelial cancers. The MTA1 protein may prove to be an important regulatory protein that is altered in its amounts in metastatic epithelial cells.

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