Metastasis-associated Protein 1 Drives Tumor Cell Migration and Invasion through Transcriptional Repression of RING Finger Protein 144A*§

Received for publication, October 14, 2011, and in revised form, December 16, 2011. Published, JBC Papers in Press, December 19, 2011, DOI 10.1074/jbc.M111.314088

Hezlin Marzook†1, Da-Qiang Li†1, Vasudha S. Nair†, Prakrit Mudvari†, Sirigiri Divijendra Natha Reddy†, Suresh B. Pakala†, T. R. Santhoshkumar‡, M. Radhakrishna Pillai2,3, and Rakesh Kumar1,3

From the †Cancer Research Program, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram 695014, India and the §Department of Biochemistry and Molecular Biology, School of Medicine and Health Sciences, George Washington University, Washington, D. C. 20037

Background: The mechanistic role of MTA1 in tumor aggressiveness is yet to be deciphered.

Results: RNF144A is a direct target of transcriptional repression by MTA1 and inhibits migration and invasion.

Conclusion: Transcriptional repression of RNF144A by MTA1 confers a migratory and invasive phenotype of cancer cells.

Significance: This study provides novel mechanistic insights into regulation of tumor progression by MTA1.

Metastasis-associated protein 1 (MTA1), a component of the nucleosome-remodeling and histone deacetylase complex, is widely up-regulated in human cancers and significantly correlated with tumor invasion and metastasis, but the mechanisms involved remain largely unknown. Here, we report that MTA1 transcriptionally represses the expression of RING finger protein 144A (RNF144A), an uncharacterized gene whose product possesses potential E3 ubiquitin ligase activity, recruiting the histone deacetylase 2 (HDAC2) and c/EBPα co-suppressor complex onto the RNF144A promoter. Functional correlation between the expression levels of MTA1 and RNF144A was demonstrated in publicly available breast cancer microarray datasets and the MCF10AD breast cancer progression model system. To address functional implications of the repression of RNF144A, we demonstrate that MTA1-mediated transcriptional repression is a critical regulatory mechanism of cancer migration and invasion. These results suggest that RNF144A is partially responsible for MTA1-mediated tumor cell migration and invasion and that MTA1 overexpression in highly metastatic cancer cells drives cell migration and invasion by, at least in part, interfering with the suppressive function of RNF144A through transcriptional repression of RNF144A expression. Together, these findings provide novel mechanistic insights into regulation of tumor progression and metastasis by MTA1 and highlight a previously unrecognized role of RNF144A in MTA1-driven cancer cell migration and invasion.

Although advances have been made in tumor diagnosis and therapies, over 90% of cancer-associated mortality is attribut-
The MTA1-RNF144A Pathway in Tumor Migration and Invasion

The MTA1-RNF144A Pathway in Tumor Migration and Invasion

(16, 17). RNF144A is an uncharacterized gene whose encoding protein contains a RING finger motif that is known to be involved in protein-protein interaction as well as protein ubiquitination and degradation (18). As many RING finger proteins, such as breast cancer type 1 susceptibility protein (BRCA1; also known as RING finger protein 53), are implicated in cancer development and progression (19), in the present study we attempted to detail the regulatory mechanism of RNF144A by MTA1 and to investigate the functional implication of the MTA1-RNF144A pathway in cancer progression.

Here, we provide convincing evidence that MTA1 transcriptionally represses RNF144A expression by recruiting the histone deacetylase 2 (HDAC2) and CCAAT/enhancer-binding protein α (c/EBPα) co-repressor complex onto human RNF144A promoter. Moreover, we demonstrate that RNF144A is a novel suppressor of cancer migration and invasion. Consequently, MTA1 overexpression in highly metastatic tumor cells enhances the ability of cancer cells to migrate and invade by, at least in part, interfering with RNF144A function through transcriptional repression of RNF144A. Thus, the dysregulated MTA1-RNF144A pathway is intimately linked to the migratory and invasive phenotype of cancer cells and may represent a promising target for cancer therapy.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—Human MCF-7 breast carcinoma cells and human HeLa epithelial cervical carcinoma cells were obtained from American Type Culture Collection (Manassas, VA). Wild-type (MTA1+/+) mouse embryonic fibroblast (MTA1−/−) MEFs were generated from MTA1−/− embryos at day 9 of development. Human colon cancer cells (HCT116) and immortalized normal breast epithelial MCF10A, pre-malignant MCF10AC1 and ductal carcinoma in situ MCF10DCIS, metastatic MCF10AC1D cell lines have been described previously (21–25) and were cultured in DMEM/F-12 medium supplemented with 5% horse serum, 10 ng/ml epidermal growth factor, 500 ng/ml hydrocortisone, 100 ng/ml cholera toxin, and 10 μg/ml insulin. Stable clones of MCF-7 cells expressing pcDNA3.1 empty vector (MCF-7/pcDNA) and T7-MTA1 (MCF-7/T7-MTA1) were established in our laboratory (26) and maintained in DMEM/F-12 medium containing 200 μg/ml Geneticin (G418). All of the cell lines used were incubated in a humidified 5% CO2 chamber at 37 °C, and all cell culture reagents were purchased from Invitrogen (Carlsbad, CA) unless specifically stated otherwise.

Antibodies, Western Blotting Analysis, and Immunoprecipitation—Rabbit polyclonal anti-MTA1 (BL1805) and anti-T7 antibodies were purchased from Bethyl Laboratories (Montgomery, TX). Rabbit polyclonal anti-HDAC2 (H-54) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology). Rabbit monoclonal anti-c/EBPα (EP709Y) and rabbit polyclonal anti-RNF144A antibodies were obtained from Abcam (Cambridge, MA). Mouse monoclonal anti-β-actin (AC40) and anti-vinculin (hVIN-1) were obtained from Sigma-Aldrich. Horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemiluminescence (ECL) reagents were purchased from Amersham Biosciences (Piscataway, NJ). All of the primary antibody dilutions were used according to manufacturer’s instructions, and all reagents were obtained from Sigma-Aldrich unless otherwise stated.

Protein extracts were prepared by lysing the cells with RIPA (radio-immunoprecipitation assay) buffer containing 50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1× protease inhibitor mixture (Roche Applied Science, Indianapolis, IN), and 1× phosphatase inhibitor mixture I and II (Sigma–Aldrich), and protein concentrations were determined using Bio-Rad DC Protein Assay reagents (Bio-Rad). Cell extracts were then resolved by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with the indicated antibodies. Corresponding antibody specific signal detections were performed using the ECL reagents and protein bands intensities were quantified using NIH image processing software (ImageJ) following manufacturer’s instructions. The quantification of Western blot signal, a set area around the band of interest was selected, the average pixel intensity was measured, and the results were normalized to the loading control band. For quantification of Western blot signal, a set area around the band of interest was selected, the average pixel intensity was measured, and the results were normalized to the loading control band. Antibodies were detected using the appropriate secondary antibodies and enhanced chemiluminescence reagents. Proteins were then transferred to nitrocellulose membranes and incubated with the indicated antibodies. The bands were visualized using enhanced chemiluminescence reagents.

For immunoprecipitation (IP) analysis of the interaction of MTA1 with c/EBPα, nuclear extracts were prepared from T7-MTA1 MEFs and precipitated with total 30 μl of Trueblot IP beads (eBioscience, San Diego, CA) for 2 h at 4 °C. The immunoprecipitates were collected by centrifugation in a microcentrifuge at 4,000 rpm for 5 min, and then the supernatant was discarded, whereupon the pellet was washed with buffer E (20 mM HEPES, pH7.9, 1.5 mM MgCl2, 150 mM KCl, 0.2 mM EDTA, 150 mM NaCl, 1 mM EDTA, 1× phosphatase inhibitor mixture I and II) on ice for 1 h with intermittent tapping for efficient lysis. Nuclear lysates were harvested by centrifugation at 14,000 rpm for 20 min at 4 °C and diluted with buffer D (buffer C without KCl) to dilute KCl concentration to final working concentration of 150 mM. The diluted fraction was incubated with 1 μg of primary antibody and corresponding control IgG overnight at 4 °C on a rocker platform, followed by incubation with total 30 μl of Trueblot IP beads (eBioscience, San Diego, CA) for 2 h at 4 °C. The immunoprecipitates were collected by centrifugation in a microcentrifuge at 4,000 rpm for 5 min, and then the supernatant was discarded, whereupon the pellet was washed with buffer E (20 mM HEPES, pH7.9, 1.5 mM MgCl2, 150 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 25 mM glycerol, 1× protease inhibitor mixture, and 1× phosphatase inhibitor mixture I and II) for three to five times and then dissolved in a sample buffer for review.
SDS-PAGE. To reduce interference by the ~55 kDa heavy and ~23 kDa light chains of the immunoprecipitating antibody, HRP-conjugated TrueBlot secondary antibodies (eBioscience) were used in the IP/immunoblotting applications.

Quantitative Real-time PCR (qRT-PCR)—Total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol, and 2 μg of extracted RNA was converted to cDNA using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). The resultant cDNA was subjected to qRT-PCR by using the iQ™ SYBR® Green Supermix (Bio-Rad) on a CFX96™ Real-Time PCR Detection System (Bio-Rad). The values for specific genes were normalized to β-actin as a housekeeping control gene. Mean values are displayed as ± standard deviations. All of qRT-PCR primers were synthesized by Sigma-Aldrich, and the sequences of the primers are available in the Table S1.

siRNA and Transfections—Specific siRNAs targeting human MTA1 and RNF144A were purchased from Dharmacon (Lafayette, CO), and the specificity of these siRNA has been verified previously (28). The transfection of siRNA was performed twice at 24-h intervals with Oligofectamine™ reagent (Invitrogen) according to the manufacturer’s protocol. Cells were subjected to further analyses after 48 h of second round of transfection.

Cloning of Human RNF144A Promoter Construct, Luciferase, and β-Galactosidase Assays—Human RNF144A promoter construct (~2130 to −1613) was amplified from genomic DNA using primers CTTTGTATTTTAGCAGCTT (forward) and GCCAGCTTTTCCTGAGCAT (reverse), and the resulting fragment was cloned into the pGL3 basic vector using In-Fusion Dry-Down PCR Cloning kit (Clontech, Mountain View, CA). The sequence was confirmed by DNA sequencing.

Plasmid DNA was purified using Qiagen Plasmid Midi Kit (Qiagen, Valencia, CA), and transfections were performed using FuGENE HD Transfection Reagent (Roche Applied Science, Indianapolis, IN) according to manufacturer’s instructions. Briefly, cells were seeded in six-well plates 24 h prior to transfection, and transfected with the indicated expression vectors. The mammalian reporter pCMVβ vector expressing extremely high levels of β-galactosidase from the human cytomegalovirus immediate early promoter (Clontech, Mountain View, CA) was used as an internal control for transfection efficiency. The pGL3 basic vector (Promega) was used as a negative control. Cells were harvested after 48 h of transfection, and luciferase as well as β-galactosidase activities were determined using the Luciferase Assay System (Promega) on a luminometer according to the manufacturer’s instructions. Luciferase activity was normalized for β-galactosidase activity in cell lysate and expressed as average of three independent experiments. Mean values are displayed as ± S.D., and statistical analysis was performed using a Student’s t test to calculate the significance of the difference between different experimental conditions.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared using a Nonidet P-40 lysis method (27) as described above and 2 μg of nuclear extracts was used for each reaction. EMSA for RNF144A DNA binding was performed using the annealed and [γ-32P]ATP end-labeled oligonucleotides or PCR product in a 20 μl reaction mixture for 15 min at 20 °C. Samples were run on a non-denaturing 5% polyacrylamide gel and imaged by autoradiography. Specific competitors were performed by adding a 100-fold excess of cold probe to the incubation mixture, and supershift EMSAs were performed by adding 100 ng of either c/EBPs or MTA1 antibodies.

Chromatin Immunoprecipitation (ChIP) Assay—The ChIP assays were performed as described previously (26). Briefly, ~106 cells were treated with 1% formaldehyde (final concentration) for 10 min at 37 °C to cross-link histones to DNA and then washed twice with phosphate-buffered saline containing protease inhibitors mixture. Cells were lysed by sonication on ice and centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatant was immunoprecipitated with specific antibodies (1 μg of primary antibody per 1 μg of protein extracts) and corresponding IgG controls. After the immunoprecipitates were washed twice, the DNA was eluted off the beads, and purified DNA (phenol-chloroform extracted) was subjected to PCR analysis. PCR primers for ChIP experiments are listed in supplemental Table S2.

Wound-healing Assay—For wound-healing assays, the V7/17-MTA1 stable clone 106 cells were treated with 1% formaldehyde for 24 h and then transfected with siRNA targeting human MTA1. After 24 h of transfection, each plate was received multiple “wounds” with a 200 μl pipette tip. The distances required to calculate the width of the wound were measured at 0 h and 24 h, and each well was examined by phase-contrast microscopy for the amount of wound closure by measuring the physical separation remaining between the original edges. The wound widths using the Olympus DP2-BSW digital camera software (Olympus, Center Valley, PA). Ten separate measurements were made per plate, and each experiment was performed in triplicates.

For invasion assay, after 24 h of siRNA transfection, cells were trypsinized, washed with PBS, and resuspended in DMEM medium containing 0.1% bovine serum albumin. Then, cells were loaded onto the upper well of a matrigel coated Boyden chamber (BD Biosciences) at a concentration of 1 × 105 cells per well. The lower side of the separating filter was filled with conditioned medium prepared from NIH3T3 cells which serves as a source of chemoattractant. Cells were stained with DAPI, and imaged using Olympus IX71 inverted microscope with DP2-BSW application software (Olympus Imaging America Inc., Center Valley, PA). Cell numbers for invasion were then determined by counting the number of cells present in 15 microscope fields at 20× magnification per insert.

Data Mining—Two publicly available microarray datasets containing primary breast tumor samples GSE22093 (29) and GSE3494 (30) were used for analyzing the correlation between MTA1 and RNF144A expression levels. They can be accessed from Gene Expression Omnibus (GEO). The probes used to detect the transcript levels of MTA1 were 202247_s_at and 211783_s_at and that used to detect RNF144A levels was 204040_at. The intensity values for each of the probes were summarized and normalized with GeneSpring GX 11.0 using RMA algorithm. The log transformed values for each probe
were then averaged and the mean was used to analyze the correlation between MTA1 and RNF144A in the chosen dataset. Based on the mean expression levels of MTA1, patients were divided into three groups. The patients with mean value of MTA1 higher than mean ± S.D. were defined as MTA1 high expression group, which lower than mean ± S.D. were defined as MTA1 low expression group, others patients were defined as medium expression group. Student’s t test was used to compare the RNF144A transcript levels in patients expressing higher and lower MTA1 levels. A p value of less than 0.05 was considered as significant in all the analyses performed.

RESULTS

Bioinformatic Analysis of the Correlation of the Transcript Levels between MTA1 and RNF144A in Multiple Published Datasets—We have shown previously that MTA1 has an oblig-atory role in regulating RNF144A expression in a p53-independent manner using microarray-based expression profiling analysis (16). To further validate the significance of these findings, we next analyzed correlation of the transcription levels of MTA1 and RNF144A in two publicly available microarray data-sets containing primary breast tumor samples (GSE22093 and GSE3494), and found a significant negative correlation between transcript levels of MTA1 and RNF144A (Fig. 1, A and B). Student’s t test analysis showed a significant difference between the means of RNF144A transcript levels in samples expressing lower and higher levels of MTA1 mRNA levels (Fig. 1, C and D). The mean RNF144A expression in samples containing low MTA1 level was found to be higher than mean RNF144A mRNA level in samples with high MTA1 levels (Fig. 1, C and D). These results demonstrate an inverse correlation of the transcript levels between MTA1 and RNF144A in multiple microarray datasets.

Expression Patterns of Endogenous MTA1 and RNF144A in a Well Established Breast Cancer Progression Model System—To demonstrate the physiological significance of MTA1 regulation of RNF144A, we next analyzed the expression levels of endog-enous MTA1 and RNF144A proteins in a well-established breast cancer progression model system, including the non-malignant MCF10A, weakly tumorigenic MCF10AT, highly proliferative and invasive MCF10DCIS, and undifferentiated metastatic MCF10CA1D cell lines (21–25). We found that the protein levels of MTA1 were up-regulated, while the levels of RNF144A protein were gradually down-regulated from non-malignant (lane 1) to highly metastatic (lane 4) breast cancer cell lines (Fig. 2, A and B). Following these observations, we next investigated whether the change of MTA1 and RNF144A in protein levels is resultant of increased corresponding mRNA levels. qRT-PCR analysis demonstrate that the expression of RNF144A and MTA1 mRNA levels followed a reverse correlation in the MCF10 progression panel cell lines corresponding to
their metastatic potential (Fig. 2C). Together, these results suggest a reverse correlation of MTA1 and RNF144A in human breast cancer progression model system, highlighting a potential role of MTA1-RNF144A pathway in tumorigenesis and tumor progression.

MTA1 Regulates RNF144A Expression at mRNA Levels—We next evaluated the status of RNF144A expression under the condition of MTA1 up-regulation or knockdown to test whether MTA1 could regulate RNF144A expression. In agreement with the above observations, we found that forced expression of MTA1 in MCF-7 stable clone cells (26) led to a significant decrease in the RNF144A protein expression (Fig. 3, A and B). As human HeLa cells express high levels of endogenous MTA1 protein according to the results from our laboratory (31) and others (32), we next knocked down endogenous MTA1 in HeLa cells using a specific MTA1 siRNA (28) to verify that the observation of an inverse correlation of MTA1 and RNF144A is general but not unique phenomenon in MCF-7 cells only. As shown in Fig. 3, C and D, knockdown of endogenous MTA1 resulted in an increase in the protein levels of RNF144A in MTA1 siRNA-transfected cells as compared with control siRNA-treated cells with a given degree of MTA1 knockdown. These results suggest that MTA1 negatively regulates RNF144A expression.

To figure out the mechanism of MTA1 regulation of RNF144A, we next examined the possibility that MTA1 affects the mRNA levels of RNF144A. In support of this notion, we found that MTA1 overexpression in MCF-7 cells decreases (Fig. 3E), whereas MTA1 silencing by siRNA in HeLa cells increases (Fig. 3F), the mRNA levels of RNF144A. Moreover, we further demonstrate that Actinomycin-D (Act-D), a putative transcriptional inhibitor (33, 34), effectively blocked MTA1-mediated repression of RNF144A mRNA levels (Fig. 3G), suggesting that MTA1 regulates RNF144A expression, at least in part, at transcriptional level.

MTA1 Is Recruited onto Human RNF144A Promoter and Inhibits Its Promoter Activity—To gain a deeper insight into MTA1 regulation of RNF144A transcription, we next examined the recruitment of MTA1 onto human RNF144A promoter using a ChIP-based promoter-walk assay (35). We found that MTA1 was recruited onto regions R2 (31002 to 906), R3 (1366 to 1124), R4 (1788 to 1613), R5 (2130 to 1953), and R6 (2914 to 2668) of human RNF144A promoter in MCF-7 cells (Fig. 4, A and B). Given the fact that MTA1 is a dual-functional transcriptional co-regulator, which can not directly bind to DNA, and that MTA1 interacts with histone deacetylase 2 (HDAC2) and both MTA1 and HDAC2 are components of the NuRD co-repressor complex (26, 36), we next tested whether HDAC2 and MTA1/HDAC2 complex were recruited to human RNF144A promoter. Indeed, ChIP assay demonstrated that HDAC2 alone (Fig. 4C) and the MTA1/HDAC2 complex (Fig. 4D) were recruited to the regions...
The MTA1-RNF144A Pathway in Tumor Migration and Invasion

In light of these findings, human RNF144A contains two regions (R4 and R5; −2130 to −1613) within the RNF144A promoter that may interact with specific proteins including MTA1. As shown in Fig. 4E, we found a significantly increased RNF144A promoter activity in the MTA1-knock-out (MTA1−/−) MEFs relative to its wild-type (MTA1+/+) controls. In contrast, the RNF144A promoter activity was reduced in the MTA1-overexpressing MCF-7 stable clone cells (MCF-7/T7-MTA1) (26) as compared with its empty vector-expressing controls (MCF-7/pDNA) (Fig. 4F). These results collectively suggest that MTA1 is recruited to the human RNF144A promoter and inhibits its promoter activity, and further strengthen the notion that MTA1 regulates RNF144A at least in part at transcriptional level.

Recruitment of the MTA1/HDAC2/c/EBPα Co-repressor Complex onto Human RNF144A Promoter—Because MTA1 is a transcriptional coregulator that interacts with transcription factors to either activate or repress the transcription of specific genes (36), we next performed a bioinformatic analysis using the AliBaba 2.1 software program to examine the nature of the putative transcription factor binding sites in the MTA1/HDAC2 complex-RNF144A promoter interacting regions (−2130 to −1613). Interestingly, we found 2 putative consensus binding sequences for the CCAAT/enhancer binding protein α (c/EBPα) transcription factor in these regions (Fig. 5A).

We next tested the hypothesis that MTA1 is a transcriptional co-repressor of RNF144A gene through the recruitment of c/EBPα transcription factor onto RNF144A promoter. Indeed, ChIP assay revealed that c/EBPα was recruited to only region R4 (−1788 to −1613) of RNF144A promoter (Fig. 5B). Moreover, the results of a double ChIP assay, in which the initial ChIP analysis was performed with an anti-MTA1 antibody followed by the second ChIP analysis using an anti-c/EBPα antibody, demonstrate that the MTA1/c/EBPα complex was co-recruited onto region R4 (−1788 to −1633) of human RNF144A promoter (Fig. 5C). Given the fact that HDAC2 interacts with c/EBPα (37), we next showed that the HDAC2/c/EBPα complex was also recruited to the same region (Fig. 5D).

These findings were further confirmed by electrophoretic mobility shift assay (EMSA) analysis using nuclear extracts from MCF-7 cells. As shown in Fig. 6A, we found that the noted protein/RNF144A DNA complex could be super-shifted by incubation of the nuclear extracts with a specific antibody against MTA1 (lane 3) or c/EBPα (lane 4) but not control IgG (lane 6) at the expense of basal protein/RNF144A DNA complexes (lane 2), suggesting that both MTA1 and c/EBPα proteins may interact with RNF144A promoter. This notion was
The MTA1-RNF144A Pathway in Tumor Migration and Invasion

The MTA1-RNF144A pathway in the migratory and invasive phenotype of cancer cells. As the biological function of RNF144A is not known so far, we first determined whether RNF144A has any role in cell migration and invasion. To this end, MCF-7 cells stably expressing empty vector (MCF-7/pcDNA) or T7-MTA1 (MCF-7/T7-MTA1) (26) were transfected with specific siRNAs targeting human RNF144A and control siRNA. As shown in supplemental Fig. S1, expression was substantially reduced in RNF144A siRNA-transfected cells as compared with control siRNA-transfected cells (Fig. 7A, upper panel). Wound-healing migration assays showed that the transfected cells exhibited larger migration gaps compared with control siRNA-transfected cells (Fig. 7B, compare column 4 with column 3). Boyden chamber invasion assays similarly indicated an increase in cell invasiveness in the RNF144A-siRNA-transfected cells as compared with control cells (Fig. 7C, upper panel). These results suggest that RNF144A, by itself, is a novel suppressor of migration and invasion (Fig. 7E).

In light of these findings, we next mutated c/EBPα consensus binding motifs in human RNF144A promoter, and demonstrated that there was a drastic reduction in the binding of the MTA1/c/EBPα complex to human RNF144A promoter DNA (Fig. 6C), indicating that the MTA1/c/EBPα complex binds to the RNF144A promoter through c/EBPα consensus binding motifs on human RNF144A promoter. Because MTA1/c/EBPα complex is recruited onto the RNF144A promoter, we next investigated the possibility of potential functional cooperation between MTA1 and c/EBPα in the regulation of the RNF144A gene transcription. As shown in Fig. 6D, we found that co-expression of MTA1 and c/EBPα (lane 4) resulted in a stronger cooperative inhibition of RNF144A promoter activity as compared with MTA1 (lane 2) or c/EBPα (lane 3) expression alone. Collectively, these results suggest that MTA1 represses RNF144A gene transcription by recruiting the MTA1/HDAC2/c/EBPα co-repressor complex onto its promoter.

Role of RNF144A in MTA1-mediated Tumor Cell Migration and Invasion—Given the fact that MTA1 has been identified as one of the critical players in cancer cell migration and invasion (10, 38, 39), we next determined the functional role of the MTA1-RNF144A pathway in the migratory and invasive phenotype of cancer cells. As the biological function of RNF144A is not known so far, we first determined whether RNF144A has any role in cell migration and invasion. To this end, MCF-7 cells stably expressing empty vector (MCF-7/pcDNA) or T7-MTA1 (MCF-7/T7-MTA1) (26) were transfected with specific siRNAs targeting human RNF144A and control siRNA. As shown in supplemental Fig. S1, expression was substantially reduced in RNF144A siRNA-transfected cells as compared with control siRNA-transfected cells (Fig. 7A, upper panel). Wound-healing migration assays showed that the transfected cells exhibited larger migration gaps compared with control siRNA-transfected cells (Fig. 7B, compare column 4 with column 3). Boyden chamber invasion assays similarly indicated an increase in cell invasiveness in the RNF144A-siRNA-transfected cells as compared with control cells (Fig. 7C, upper panel). These results suggest that RNF144A, by itself, is a novel suppressor of migration and invasion (Fig. 7E).
3, A, B, and E) and that RNF144A is a potential inhibitor of migration and invasion (above), we conclude that MTA1 overexpression in highly metastatic cancer cells facilitates the acquisition of a highly invasive and metastatic phenotype of cancer cells by, at least in part, compromising the suppressive function of RNF144A in tumor migration and invasion through transcriptional repression of RNF144A expression (Fig. 7E).

**DISCUSSION**

A critical step in the development of a metastatic tumor is characterized by a gain in the tumor cells' migratory and invasive capabilities (40). In the present study, we investigated the molecular mechanism of transcriptional regulation of RNF144A expression by MTA1 and its attributed functions in cancer migration and invasion, hallmarks of tumor metastasis in vivo. First, we made a systematic attempt to understand the correlation between the expression levels of MTA1 and RNF144A in highly metastatic cancer cells through transcriptional repression of RNF144A expression (Fig. 7E).

Although MTA1 has been shown previously to activate and repress gene transcription in the appropriate cellular and genetic context (9, 16, 36), in this study we provide the molecular details of how MTA1 drives tumor progression through the delicate transcriptional control of its target genes. Another novel finding presented here is that we demonstrate that transcriptional repression of RNF144A by MTA1 is functionally linked to the malignant phenotype of cancer cells. In this context, we discovered that siRNA-mediated knockdown of human RNF144A, which had not previously been implicated in cancer, increased the migration and invasiveness of MCF-7 cells, suggesting that RNF144A is a novel suppressor of tumor progression.
migration and invasion (Fig. 7). This novel finding supports the observations that the expression levels of RNA144A are negatively correlated with those of MTA1 in various tumor models (Figs. 1 and 2). Although the mechanism by which RNF144A suppresses the migratory and invasive ability of MCF-7 cells is currently unknown, the mechanistic role of other members of the RNF gene family in cancer invasion and metastasis has been documented. For example, RING finger protein 5 (RNF5) has been shown to inhibit cell motility by targeting cytoskeletal protein paxillin ubiquitination and altered localization (41). In contrast, RNF13 and RNF55 (also known as proto-oncogene c-Cbl) promote pancreatic cancer and glioma invasion by enhancing the activity of extracellular matrix metalloproteinase-9 (MMP-9) and MMP-2, respectively (42, 43), which are required for degrading structural extracellular matrix proteins to promote invasion and metastasis (44). Similarly, RNF45 (also known as tumor autocrine motility factor receptor or gp78) promote sarcoma metastasis by targeting the metastasis suppressor KA11 for degradation (45). Originally, RNF45 was isolated as a membrane glycoprotein from murine melanoma cells and was implicated in cell migration (46, 47). Subsequent studies identified RNF45 as the tumor autocrine motility factor receptor mediating tumor invasion and metastasis (47, 48). In addition, expression of RNF188 (also known as HAKAI or CBLL1) also increases epithelial cell invasion (49). Given that many RING finger proteins have intrinsic E3 ligase activities (18), it is possible that RNF144A exerts its effect on cell migration and invasion through ubiquitylating, possibly causing degradation of key effector proteins of the migration/invasion machinery.

In support of its promoting role in metastasis (10–12), we demonstrated that MTA1 expression enhances cell migration and invasion ability in MCF-7 cells (Fig. 7). More interestingly, we demonstrated that siRNA-mediated knockdown of endogenous RNF144A accelerates the motility and invasion of MTA1-overexpressing cells, suggesting that the effect of MTA1 on cell migratory and invasive functions is dependent on RNF144A expression. Thus, MTA1 facilitates the acquisition of an invasive and metastatic phenotype of cancer cells by, at least in part, compromising the suppressive function of RNF144A in tumor migration and invasion through transcriptional repres-
The MTA1-RNF144A Pathway in Tumor Migration and Invasion

In summary, we demonstrate that RNF144A is a direct target of transcriptional repression by MTA1 and is a novel potential inhibitor of cell migration and invasion. MTA1 overexpression in highly metastatic tumor cells inhibits RNF144A expression and function, facilitating the acquisition of a highly invasive and metastatic phenotype of cancer cells. In addition, as RNF144A protein contains a putative RING finger domain and MTA1 is an ubiquitinated protein (50), it will be interesting to determine whether MTA1 would be targeted by RNF144A as a potential E3 ubiquitin-protein ligase for the ubiquitin-dependent degradation. Thus, MTA1 and RNF144A may form a double vicious circle, contributing to tumor aggressiveness and progression. Collectively, the results presented here provide novel mechanistic insights into regulation of tumor progression by MTA1 and highlight a previously unrecognized role of RNF144A in MTA1-driven cancer cell migration and invasiveness. These findings open the possibility that development of specific modifiers of the MTA1-RNF144A pathway may lead to novel therapeutic approaches for targeting the metastatic process in the future.

Acknowledgments—We thank all the members of the Kumar laboratory for technical assistance and fruitful discussion and are grateful to Dr. Lei Wang (Department of Pathology and Immunology, Baylor College of Medicine, Houston, TX) for microarray data mining.
The MTA1-RNF144A Pathway in Tumor Migration and Invasion

REFERENCES

1. Valastyan, S., and Weinberg, R. A. (2011) Tumor metastasis: molecular insights and evolving paradigms. Cell 147, 275–292
2. Gupta, G. P., and Massagué, J. (2006) Cancer metastasis: building a framework. Cell 127, 679–695
3. Steeg, P. S. (2006) Tumor metastasis: mechanistic insights and clinical challenges. Nat. Med. 12, 895–904
4. Wang, W., Eddy, R., and Condeelis, J. (2007) The cofilin pathway in breast cancer invasion and metastasis. Nat. Rev. Cancer 7, 429–440
5. Toh, Y., and Nicolson, G. L. (2009) The role of the MTA family and their encoded proteins in human cancers: molecular functions and clinical implications. Clin. Exp. Metastasis 26, 215–227
6. Kumar, R., Wang, R. A., Mazumdar, A., Talukder, A. H., Mandal, M., Yang, Z., Bagheri-Yarmark, R., Sahin, A., Hemborg, G., Adam, L., Barnes, C. J., and Vladiamari, R. K. (2002) A naturally occurring MTA1 variant sequesters estrogen receptor-α in the cytoplasm. Nature 418, 645–657
7. Toh, Y., Pencil, S. D., and Nicolson, G. L. (1994) A novel candidate metastasis-associated gene, mta1, differentially expressed in highly metastatic mammmary adenocarcinoma cell lines. cDNA cloning, expression, and protein analyses. J. Biol. Chem. 269., 22958–22963
8. Kumar, R., Wang, R. A., and Bagheri-Yarmark, R. (2003) Emerging roles of MTA family members in human cancers. Semin Oncol. 30, 30–37
9. Manavathi, B., Singh, K., and Kumar, R. (2007) MTA family of coregulators in nuclear receptor biology and pathology. Nucl. Recept. Signal 5, e010
10. Mahoney, M. G., Simpson, A., Jost, M., Noé, M., Kari, C., Pepe, D., Choi, Y. W., Uitto, J., and Rodeck, U. (2002) Metastasis-associated protein (MTA1) enhances migration, invasion, and anchorage-independent growth of immortalized human keratinocytes. Oncogene 21, 3128–3139
11. Nicolson, G. L., Nawa, A., Toh, Y., Taniguchi, S., Moustafa, A. (2003) Tumor metastasis-associated gene, mta1, differentially expressed in highly metastatic B16F10 melanoma cells. J. Exp. Clin. Cancer Res. 22, 13128–13133
12. Hofer, M. D., Menke, A., Genze, F., Gierschik, P., and Giehl, K. (2004) Targeting prostate cancer angiogenesis through metastasis-associated gene 1 inhibition in a C57BL/6 mouse model. Prostate 61, 1475–1489
13. Qian, H., Yu, J., Li, Y., Wang, H., Song, C., Zhang, X., Liang, X., Fu, M., and Kumar, R. (2011) Metastasis-associated gene 1 influences on protein expression of ERα in MCF10A and MCF10AT cells. Breast Cancer Res. Treat. 127, 101–110
14. Kai, L., Wang, J., Ivanovic, M., Chung, Y. T., Laskin, W. B., Schulze-Hoepfner, F., Mirochnik, Y., Satcher, A., and Stevenson, A. S. (2011) Targeting prostate cancer angiogenesis through metastasis-associated protein 1 (MTA1). Prostate 71, 268–280
15. Jiang, Q., Zhang, H., and Zhang, P. (2011) shRNA-mediated gene silencing of MTA1 influenced on protein expression of ERα, MMP-9, CyclinD1 and invasion, proliferation, in breast cancer cell lines MDA-MB-231 and MCF-7 in vitro. J. Exp. Clin. Cancer Res. 30, 60
16. Ghanta, K. S., Eswaran, J., and Kumar, R. (2011) Gene profiling of MTA1 identifies novel gene targets and functions. PLoS One 6, e17135
17. Li, D. Q., Pakala, S. B., Reddy, S. D., Ohshiro, K., Peng, S. H., Lian, Y., Fu, S. X., and Kumar, R. (2010) Revelation of p53-independent function of MTA1 in DNA damage response via modulation of the p21 WAF1-protein 1-alternative reading frame pathway in oncogenesis. Mol. Cell Biol. 30, 5639–5647
18. Khaleque, M. A., Bharti, A., Gong, J., Gray, P. I., Sachdev, V., Ciocca, D. R., Stati, A., Fanelli, M., and Calderwood, S. K. (2008) Heat shock factor 1 represses estrogen-dependent transcription through association with MTA1. Oncogene 27, 1886–1893
19. Sober, H. M. (1985) Actinomycin and DNA transcription. Proc. Natl. Acad. Sci. U.S.A. 82, 5328–5331
20. Flamée, P. A. (1985) The action of actinomycin D on the transcription of c/EBP-β and c/EBP-α in rat liver. J. Biol. Chem. 260, 10044–10052
21. Qian, H., Lu, N., Xue, L., Liang, X., Zhang, X., Fu, M., Xie, Y., Zhan, Q., Liu, Z., and Lin, C. (2005) Reduced MTA1 expression by RNAi inhibits migration, invasion and anchorage-independent growth of cervical cancer cells in vitro. J. Biol. Chem. 280, 230, 5625–5630
22. Li, D. Q., Pakala, S. B., Reddy, S. D., Ohshiro, K., Zhang, J. X., Wang, L., Zhang, Y., Moreno de Albornoz, I., Pillai, M. R., Eswaran, J., and Kumar, R. (2011) Bidirectional autoregulatory mechanism of metastasis-associated protein-1-alternative reading frame pathway in oncogenesis. Proc. Natl. Acad. Sci. U.S.A. 108, 8791–8796
23. Manavathi, B., and Kumar, R. (2007) Metastasis tumor antigens, an emerging family of multifaceted master coregulators. J. Biol. Chem. 282, 1529–1533
24. Duan, H., Heckman, C. A., and Boxer, L. M. (2005) Histone deacetylase inhibitors down-regulate bcl-2 expression and induce apoptosis in (14; 18) lymphomas. Mol. Cell Biol. 25, 1608–1619
25. Qian, H., Lu, N., Xue, L., Liang, X., Zhang, X., Fu, M., Xie, Y., Zhan, Q., Liu, Z., and Lin, C. (2005) Reduced MTA1 expression by RNAi inhibits in vitro invasion and migration of esophageal squamous cell carcinoma cell line. Clin. Exp. Metastasis 22, 653–662
26. Hao, Y., Wang, H., Fan, L., and Chen, G. (2011) Silencing MTA1 by RNAi reverses adhesion, migration and invasiveness of cervical cancer cells (SiHa) via altered expression of p53, and E-cadherin/β-catenin complex. J. Huazhong. Univ. Sci. Technolog. Med. Sci. 31, 1–9
27. Wicki, A., Lehembre, F., Wick, N., Hantusch, B., Kerjaschki, D., and Christofori, G. (2006) Tumor invasion in the absence of epithelial-mesenchymal
mal transition: podoplanin-mediated remodeling of the actin cytoskeleton. *Cancer Cell* 9, 261–272
41. Didier, C., Broday, L., Bhoumik, A., Israeli, S., Takahashi, S., Nakayama, K., Thomas, S. M., Turner, C. E., Henderson, S., Sabe, H., and Ronai, Z. (2003) RNF5, a RING finger protein that regulates cell motility by targeting paxillin ubiquitination and altered localization. *Mol. Cell Biol.* 23, 5331–5345
42. Zhang, Q., Meng, Y., Zhang, L., Chen, J., and Zhu, D. (2009) RNF13: a novel RING-type ubiquitin ligase overexpressed in pancreatic cancer. *Cell Res.* 19, 348–357
43. Lee, H., and Tsygankov, A. Y. (2010) c-Cbl regulates glioma invasion through matrix metalloproteinase 2. *J. Cell Biochem.* 111, 1169–1178
44. Egeblad, M., and Werb, Z. (2002) New functions for the matrix metalloproteinases in cancer progression. *Nat. Rev. Cancer* 2, 161–174
45. Tsai, Y. C., Mendoza, A., Mariano, J. M., Zhou, M., Kostova, Z., Chen, B., Veenstra, T., Hewitt, S. M., Helman, L. J., Khanna, C., and Weissman, A. M. (2007) The ubiquitin ligase gp78 promotes sarcoma metastasis by targeting KAI1 for degradation. *Nat. Med.* 13, 1504–1509
46. Nabi, I. R., and Raz, A. (1987) Cell shape modulation alters glycosylation of a metastatic melanoma cell surface antigen. *Int. J. Cancer* 40, 396–402
47. Fang, S., Ferrone, M., Yang, C., Jensen, J. P., Tiwari, S., and Weissman, A. M. (2001) The tumor autocrine motility factor receptor, gp78, is a ubiquitin protein ligase implicated in degradation from the endoplasmic reticulum. *Proc. Natl. Acad. Sci. U.S.A.* 98, 14422–14427
48. Nabi, I. R., Watanabe, H., and Raz, A. (1992) Autocrine motility factor and its receptor: role in cell locomotion and metastasis. *Cancer Metastasis Rev.* 11, 5–20
49. Rodríguez-Rigueiro, T., Valladares-Ayerbes, M., Haz-Conde, M., Aparicio, L. A., and Figueroa, A. (2011) Hakai reduces cell-substratum adhesion and increases epithelial cell invasion. *BMC Cancer* 11, 474
50. Li, D. Q., Ohshiro, K., Reddy, S. D., Pakala, S. B., Lee, M. H., Zhang, Y., Rayala, S. K., and Kumar, R. (2009) E3 ubiquitin ligase COP1 regulates the stability and functions of MTA1. *Proc. Natl. Acad. Sci. U.S.A.* 106, 17493–17498
In this article, the authors have inadvertently used MTA1-siRNA and control β- actin panels from other project while reprobing the same membrane for RNF144A antibody used in panel C. This change does not alter the conclusion as well as the quantification of the RNF144 protein of this study. The revised figure and figure legend are presented below.

**FIGURE 3.** MTA1 negatively regulates RNF144A expression at mRNA level. A and B, Western blot analysis of the protein extracts from MCF-7 cells stably expressing pcDNA empty vector (MCF-7/pcDNA) and T7-MTA1 (MCF-7/T7-MTA1) with the indicated antibodies (A) and quantitative results of Western blots (B) using ImageJ software. C, HeLa cells were transfected with control siRNAs or specific siRNAs targeting human MTA1. After 48 h of the second round of transfection, protein extracts were prepared and subjected to Western blot analysis with anti-RNF144A antibody. D, quantitative result of Western blot of RNF144A in panel C using ImageJ software. E and F, qRT-PCR analysis of the expression of RNF144A and MTA1 mRNA levels in the MCF-7/pcDNA and MCF-7/T7-MTA1 stable clone cells (E) and HeLa cells transfected with control siRNAs or specific siRNAs targeting human MTA1 (F). G, MCF-7 cells were transfected with control siRNAs or specific siRNAs targeting human MTA1. After 36 h of the second round of transfection, cells were treated with or without 250 ng/ml of actinomycin D (Act-D) for another 12 h and then subjected to qRT-PCR analysis of the expression of RNF144A and MTA1 mRNA levels as described above. DMSO, dimethyl sulfoxide.