MTA1 gene silencing inhibits invasion and alters the microRNA expression profile of human lung cancer cells

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Abstract. Metastasis-associated gene 1 (MTA1) is involved in the carcinogenesis and metastasis of many human carcinomas. However, its exact role in non-small cell lung cancer (NSCLC) is still unclear. Using immunohistochemistry analysis, we recently identified MTA1 to be associated with the progression of NSCLC. Here, we carried out further analysis on the effect of MTA1 knockdown in an NSCLC cell line on cell functions and the global microRNA (miRNA) expression profile. We succeeded in establishing the MTA1 knockdown NSCLC cell line using RNA interference (RNAi), and found that the silencing of MTA1 resulted in the effective inhibition of the invasive ability of NSCLC cells, but not of the cell growth *in vitro*. We performed an miRNA microarray analysis and demonstrated for the first time that MTA1 knockdown significantly changed the expression of some miRNAs in NSCLC cells. Among them, some have a well-characterized association with cancer progression, e.g. miR-125b, miR-210, miR-103, miR-194 and miR-500. In summary, it is evident from our results that MTA1 functions in regulating the invasive phenotype of lung cancer cells and this regulation may be through altered miRNA expression. The interaction between MTA1 and the miRNAs which contributes to lung cancer is worthy of further investigation.

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Introduction

Metastasis-associated gene 1 (MTA1), originally identified through differential screening of a cDNA library from rat metastatic breast tumors (1), is the founding member of the MTA family of coregulators in transcriptional programs (2), and an integral part of the nucleosome remodeling and histone deacetylation (NuRD) complex. The role of MTA1 in tumor progression has aroused wide concern. It has been found to be upregulated in human tumors (3), and to be associated with carcinogenesis and metastasis of many types of cancer, such as breast cancer, hepatocellular carcinoma, and B cell lymphomas (4-6).

In our previous study, we detected MTA1 protein expression in NSCLC tissue specimens. We found that MTA1 overexpression was significantly correlated with tumor progression and poor prognosis of NSCLC (7). However, there is still lack of direct evidence that MTA1 is responsible for aggressive phenotypes of NSCLC, and the underlying mechanism is unclear as well.

microRNAs (miRNAs) are a class of endogenous, small (21-23 nucleotides in length), non-coding RNAs. They act by regulating the expression of their target genes by perfect or imperfect complementarity, causing either mRNA molecule degradation or translational inhibition (8). Aberrant expression of miRNA is widespread in human cancers. Recent evidence indicates that miRNAs can function as either oncogenes or tumor suppressors (9,10), and play a regulatory role in the metastatic process of lung cancer (11-13). However, many cancer-related miRNAs are still unknown, and the mechanism of the miRNA involvement in tumor metastasis remains unclear. In addition, reports on the regulation of miRNA expression are scarce.

In this study, we established a stable MTA1 silencing NSCLC cell line with RNA interference (RNAi) technology, and found that silencing of MTA1 can inhibit cell invasion and migration, but has little effect on cell proliferation. We further examined the changes of the miRNA expression profile in human NSCLC 95D cells after MTA1 silencing, and the microarray results were confirmed by quantitative real-time

polymerase chain reaction analysis (qRT-PCR). Our results suggest that miRNAs may mediate, at least in part, the regulation of the metastatic phenotype of NSCLC by MTA1.

Materials and methods

Cell culture. HBE, H460, A549, and 95D cell lines grown in RPMI medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum were cultured in a humidified atmosphere in a 5% CO_2 incubator at 37˚C.

Stable transfection. The 95D cell line, which showed the maximum MTA1 expression among the cell lines examined, was selected for the MTA1 gene silencing study. Three human shRNA sequences targeting MTA1 were cloned into the pLVTHM plasmid containing the EGFP gene (Tronolab, Lausanne, Switzerland) to generate pLVTHM-MTA1-RNAi(s); the sequences were MTA1-si1#: sense, 5'-CGCGTCCCCGG AGAGATTCGAGTAGGAAACTTCAAGAGAGTTTCCTA CTCGAATCTCTCCTTTTTGGAAAT-3'; MTA1-si2#: sense, 5'-CGCGTCCCCGCAGCAGAAACGCTTGAAAG CTTCAAGCGTTTCTGCTGCTTTTTGGAAAT-3' and MTA1-si3#, sense, 5'-CGCGTCCCCAAGACCCTGCTGG CAGATAAATTCAAGAGATTTATCTGCCAGCAGGGTC TTTTTTTGGAAAT-3' (synthesized by Invitrogen). A scrambled siRNA (5'-GACGACGATAAGGGATCCTGA-3'), which has no homology with the mammalian mRNA sequences, was inserted into the pLVTHM vector as described above to generate pLVTHM-CTL-si. Cells were all transfected with 3 μg of plasmids (pLVTHM-MTA1-si1#, or pLVTHM-CTL-si), or empty pLVTHM vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The independent colonies resistant to neomycin were chosen and subcultured for 14 days in G418 selection. These three cell lines were named as 95D/MTA1-si1#, 95D/CTL-si, and 95D/NC.

Western blot analysis. Total protein was extracted from cells using RIPA kit (Pierce, USA). Protein was electrophoresed on a polyacrylamide gel and transferred to Hybond-C nitrocellulose membranes. The membranes was incubated with anti-MTA1 (Abcam, Cambridge, MA, USA), anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:1,500 dilution at 37˚C for 2 h, and then with peroxidise-conjugated goat anti-mouse IgG (Beijing Biosynthesis Biotech, Co., Ltd., Beijing, China) at 1:2,000 at room temperature for 1 h. β-actin was used as an internal control. Proteins were visualized using enhanced chemiluminescence (ECL) methods. Membranes were washed three times and then exposed to X-ray film.

Cell invasion assay. For invasion assays, $1x10^4$ cells in serum-free media were seeded into the upper chambers of a 24-well BioCoat Matrigel invasion chamber (BD Biosciences, Bedford, MA, USA) with an 8-μm pore polycarbonate membrane coated with Matrigel. Medium with 10% FBS was added to the lower chambers as a chemoattractant. After 24 h of incubation, cells remaining on the upper surface of the membrane were removed with a cotton swab, and cells that invaded through the membrane filter were fixed with 4% paraformaldehyde, stained by haematoxylin and eosin, and photographed under a microscope (Olympus BX40 with a DP70 digital camera). The number of invading cells was manually counted per high-power field for each condition (five fields on each membrane were randomly selected).

Wound healing assay. Cells were grown to confluence in a 6-well plate. Artificial wound tracks were created by scraping confluent cell monolayers with a pipette tip. After removal of the detached cells by gently washing with PBS, the cells were fed with fresh complete medium and incubated over time to allow cells to migrate into the open area. The ability of the cells to migrate into the wound area was assessed at 1, 24 and 48 h after scratching by comparing the pixels of the wound tracks in micrographs of three randomly selected wounded areas.

Cell proliferation assay and colony formation assay. The proliferation of transfected cells was measured by the MTT assay. Ten microliters of MTT (5 mg/ml) was added to each culture volume of 100 μl, which contained $1x10^3$ viable cells per well. After an additional incubation for 4 h, the resulting formazan was dissolved in 100 μl isopropanol with 40 mM hydrochloric acid. Spectrophotometric absorbance at 570 nm (for formazan dye) was measured, along with absorbance at 630 nm as a reference. To assay colony formation, a total of 1,000 cells were plated onto a 100-mm dish and cultured for 14 days in complete DMEM supplemented with blasticidine. The colonies were fixed with 100% methanol 2 x 10 min, stained with 0.2% crystal violet in 75% methanol for 30 min, and then visualized and counted.

Flow cytometry analysis. Exponentially growing cells were trypsinized, and single-cell suspensions containing $1x10^6$ cells were fixed in 70% cold ethanol at 4˚C. The cell cycle was monitored using propidium iodide (PI) staining of nuclei. The fluorescence of DNA-bound PI in cells was measured with a FACScan flow cytometer (BD Biosciences), and the results were analyzed with ModFit3.0 software (Verity Software House, Topsham, ME, USA).

miRNA microarray analysis. Total RNA samples extracted from two cell lines (95D/MTA1-si1# and 95D/NC) were analyzed by CapitalBio (CapitalBio Corp., Beijing, China) for miRNA microarray experiments. Procedures were performed as described in detail (http://www.capitalbio.com). Briefly, total RNA was extracted with TRIzol® reagent. Small-sized RNA was isolated using an miRNA isolation kit (Ambion, Inc.). Fluorescein-labeled miRNAs were then hybridized on each miRNA microarray containing 509 probes in triplicate, corresponding to 435 human (including 122 predicted miRNAs), 261 mouse and 196 rat miRNAs, to determine differential expression between the cell lines. Every sample was assayed in triplicate. Arrays were scanned with a LuxScan 10K-A laser confocal scanner and images were analyzed using LuxScan3.0 software (CapitalBio Corp.). Expression data in different chips were median centered using global median normalization. Differentially expressed miRNAs were identified by SAM, version 2.1 (Stanford University, Stanford, CA, USA). We identified genes with a false discovery rate of <5% and an absolute fold change of ≥2.0.

Table I. List of primers for qRT-PCR analysis of miRNAs (fold-change >2).

Gene	RT primers	Sequence of PCR primers (5'-3')
U6	5'-AACGCTTCACGAATTTGCGT-3'	F: CTCGCTTCGGCAGCACA R: AACGCTTCACGAATTTGCGT
hsa-miR-103	5'-extension-CATAGCC-3'	F: ACACTCCAGCTGGGAGCAGCATTGT ACAGGGC
hsa-miR-125b	5'-extension-CACAAGTT-3'	F: ACACTCCAGCTGGGTCCCTGAGACCCTAACT
hsa-miR-194	5'-extension-CCACATG-3'	F:ACACTCCAGCTGGGTGTAACAGCAACTCCATGTG
hsa-miR-210	5'-extension-CAGCCGC-3'	F: ACACTCCAGCTGGGCTGTGCGTGTGACAGCGG
hsa-miR-500	5'-extension-CTCACCC-3'	F: ACACTCCAGCTGGGTAATCCTTGCTACCTGGG
hsa-miR-744	5'-extension-GCTGTTA-3'	F: ACACTCCAGCTGGGTGCGGGGCTAGGGCTAAC
miR universal		R: CTCAACTGGTGTCGTGGA

The sequence of the 5'-extension for the RT primers is 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGT-3'.

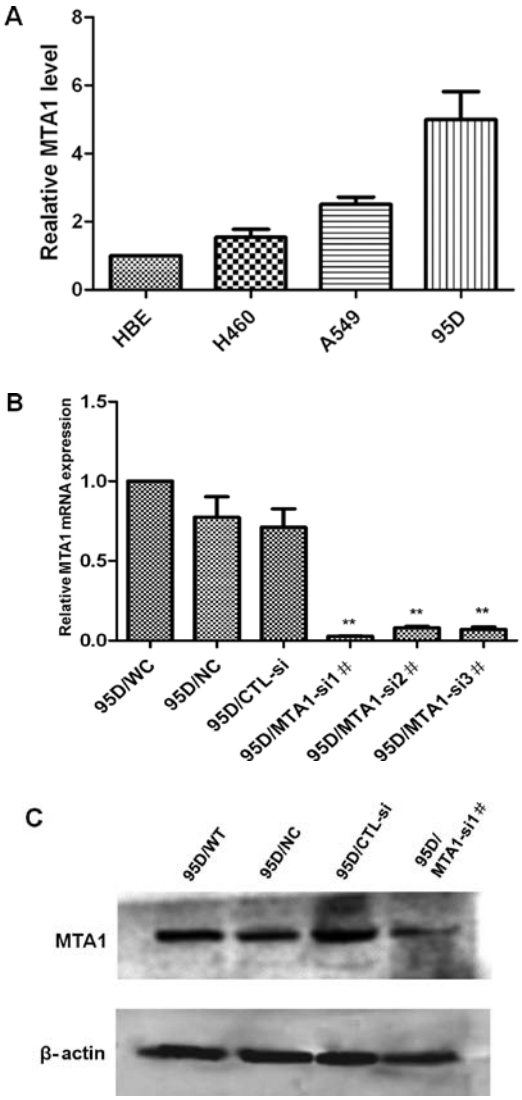

Figure 1. Expression of MTA1 in lung cancer cell lines and knockdown of MTA1 by siRNA. (A) qRT-PCR showing MTA1 mRNA levels in three lung cancer cell lines and one normal cell line. Data are expressed as a fold change relative to control (control is HBE). (B) qRT-PCR analysis of MTA1 mRNA expression in 95D/WT, 95D/NC, 95D/CTL-si and MTA1 knockdown cell lines (95D/MTA1-si1#, 95D/MTA1-si2# and 95D/MTA1-si3#). Values are given as mean ± SD of three experiments. **P<0.01 as compared to 95D/WT. (C) Western blot analysis of MTA1 protein expression in 95D/WT, 95D/NC, 95D/CTL-si and 95D/MTA1-si1# stable cell lines.

Quantitative real-time polymerase chain reaction analysis. Analysis for MTA1 mRNA was carried out as previously described (7). The primer sequences used in RT-PCR were: MTA1, forward: 5'-AGCTACGAGCAGCACAACGGGGT-3' and reverse: 5'-CACGCTTGGTTTCCGAGGAT-3'; GADPH, forward: 5'-TCTTCGCTTTGTCCTTTCGT-3' and reverse: 5'-TGCTGTAGCCAAATTCGTTG-3'.

To confirm the microarray results, total RNA was isolated as above. Reverse transcription was performed with specific primers (Table I) for hsa-miR-125b, hsa-miR-155, hsa-miR-210, hsa-miR-103, hsa-miR-194, hsa-miR-500 and hsa-miR-744, and U6 was used as an internal control. qRT-PCR was carried out using the Rotor-Gene 3000 Real-Time PCR system. The PCR reaction mixture with a total volume 25 μl contained 25 ng cDNA, 800 nM of each specific primer (Table I) and 0.25X SYBR-Green (diluted from 1000X SYBR-Green, Molecular Probes). After denaturing at 95˚C for 5 min, the reactions were amplified for 15 sec at 95˚C and 30 sec at 65˚C and 20 sec at 72˚C for 40 cycles. The thermal denaturation protocol was run at the end of the PCR to determine the number of products that were present in the reaction. The relative amount of each miRNA to U6 RNA was calculated using the $2^{-\Delta\Delta Ct}$, where Ct is the number of cycles at which amplification reaches a threshold, determined by SDS software v1.2 (Applied Biosystems Inc.).

Statistical analysis. Data analysis was performed with the SPSS 13.0 statistical software. P-values were generated by the Student's t-test for qRT-PCR data, migration assays and colony formation assays, and by two-way ANOVA for the cell proliferation assays. All experiments were performed in triplicate.

Results

Expression of MTA1 in human lung cancer cell lines and siRNA-mediated silence. First, we examined MTA1 mRNA expression in three lung cancer cell lines (H460, A549, and 95D) and the normal cell line HBE (human lung bronchial epithelium) by qRT-PCR. MTA1 was detected in all cell lines evaluated, with 95D cells expressing the highest levels (Fig. 1A). Therefore, 95D cells were selected as the model for the subsequent function studies.

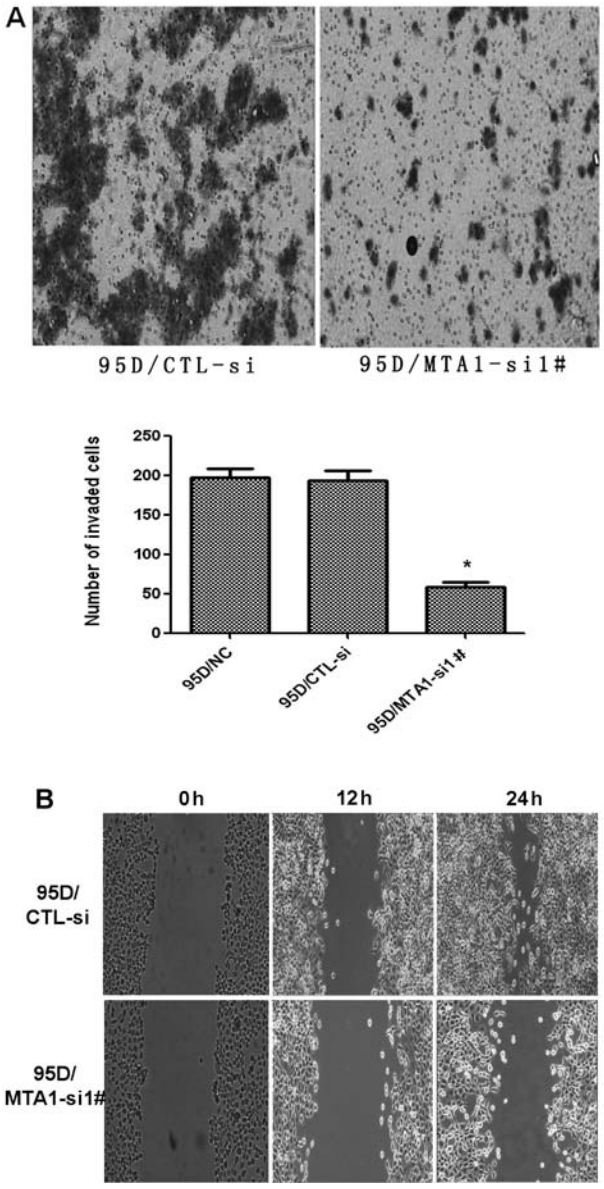

Figure 2. Silencing of MTA1 gene inhibits the migration and invasion of lung cancer cells. (A) Cell invasion assay invasion cells were fixed and stained, and representative fields were photographed. For quantification, the cells were counted in 5 random fields under a light microscope (x200). Triplicate assays were performed for each group of cells in invasion assays, and the results are expressed as means ± SD (*P<0.05). (B) Summary of wound-healing assay associated with tumor cell migration. Phase-contrast micrographs of the scratched monolayer of 95D/NC, 95D/CTL-si and 95D/MTA1-si1# cells at 0, 12 and 24 h after scratching.

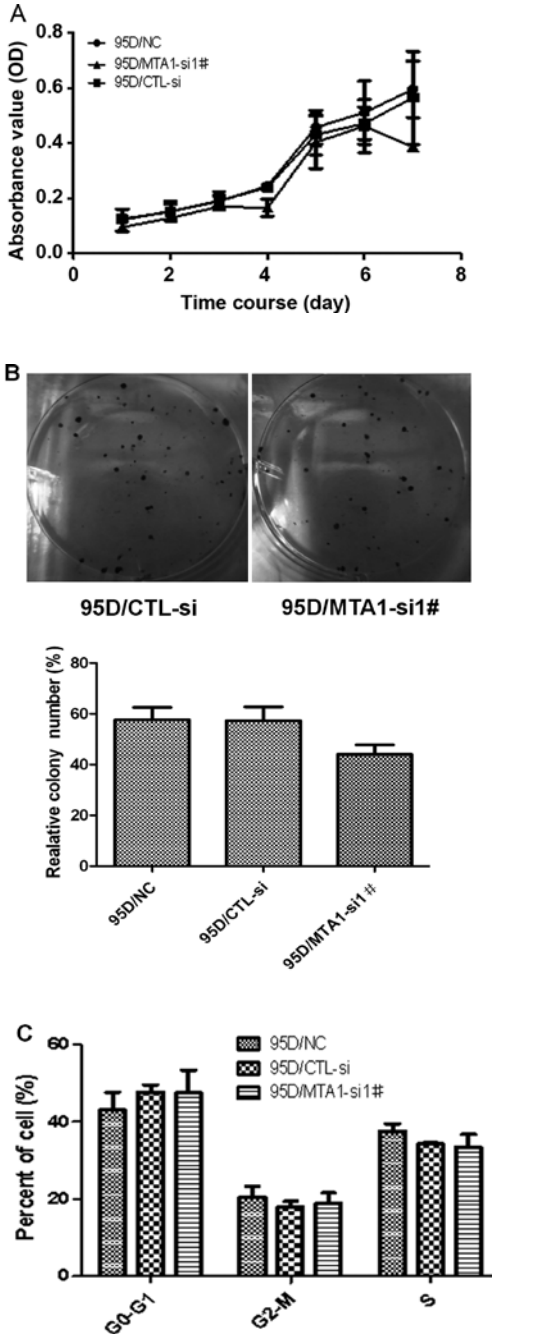

Figure 3. Silencing of the MTA1 gene has little influence on the growth of lung cancer cells. (A) MTT proliferation assay. (B) Colony formation assay. (C) Flow cytometry analysis. All results were reproducible in three independent experiments (P>0.05).

The expression of MTA1 mRNA was significantly decreased in the cells handled with the three human shRNA sequences targeting MTA1 compared to control. Furthermore, MTA1-si1# was the most effective silencing sequence (Fig. 1B). Western blot analysis showed that expression of MTA1 protein also decreased markedly in the MTA1 knockdown stable cell line 95D/MTA1-si1# (Fig. 1C).

MTA1 gene silencing inhibits 95D cell invasion and migration in vitro. We next determined the ability of 95D/MTA1-si1#, 95D/CTL-si, and 95D/NC cells to invade through extracellular matrix (ECM) in a Boyden chamber invasion assay. As shown in Fig. 2A, the number of 95D/MTA1-si1# cells invading through the membrane was significantly lower than that of 95D/NC cells (60.00±6.25 vs. 195.00±10.83, P<0.001). It appears that MTA1 affects lung cancer cell invasion.

We also used an *in vitro* wound healing assay to determine the effect of MTA1 silencing on 95D cell migration. Fig. 2B shows that the healing speed was slower and that the gaps were wider in 95D/MTA1-si1# cells at each time point than in the 95D/CTL-si cells. These data indicate that MTA1 promoted 95D cell migration.

Silencing of MTA1 gene has little influence on the growth of 95D cells. Because downregulation of MTA1 has been

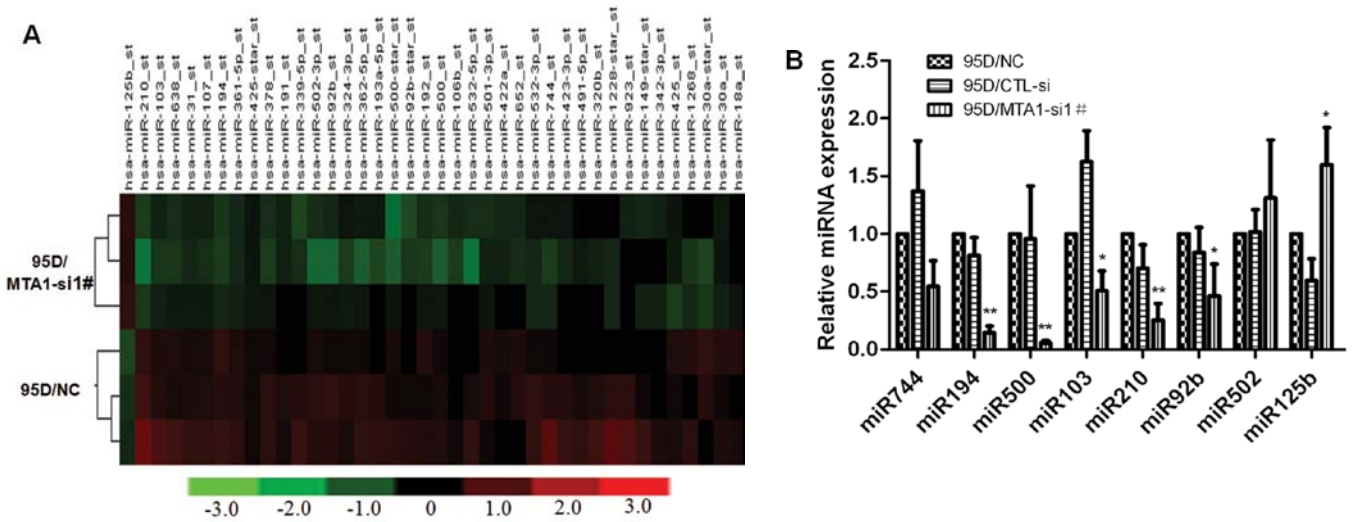

Figure 4. Effect of MTA1 gene silencing on miRNA expression profile of 95D cells. (A) Change of miRNA expression profiling of 95D cancer cells determined by microarray. Heat map of hierarchical clustering of differentially expressed miRNA in 95D/MTA1-si and 95D/NC. Red denotes high expression levels, whereas green depicts low expression levels. (B) The expression of miRNAs were verified using qRT-PCR. Values are presented as mean ± SD of three experiments ($^{*}P<0.05$, $^{**}P<0.01$ as compared to 95D/NC).

shown to inhibit breast cancer cell growth (14), we sought to examine whether silencing of MTA1 could influence 95D cell growth. Using a colony formation assay, we found that there was no significant difference in the ability to form colonies between 95D/MTA1-si1# and 95D/NC cells (Fig. 3B). The cell proliferation assay also showed little growth inhibition in 95D/MTA1-si cells compared to 95D/NC cells (Fig. 3A). These data indicate that MTA1 silencing has little effect on the growth and clonogenic capacity of 95D cells. Consistent with this result, we did not observe a difference in cell cycle progression between 95D/MTA1-si1# and 95D/NC cells (Fig. 3C).

Effect of MTA1 gene silencing on the miRNA expression profile of 95D cells. To identify the effect of MTA1 gene silencing on the miRNA expression in 95D cells, a miRNA microarray capable of measuring expression of 509 miRNAs was used to analyze the miRNA profile changes between 95D/MTA1-si and 95D/NC cell lines. The microarray analysis demonstrated that 40 miRNA genes exhibited significant differential expression (false discovery rate <5%). The relative expression of these miRNAs is presented as a heat map in Fig. 4A. Of the screened miRNAs, we only identified 6 genes with an absolute fold change >2.0. miR-125b was significantly upregulated with 2.156-fold increase in 95D/MTA1-si1# cells compared with 95D/NC cells. Contrarily, miR-210, miR-500, miR-194, miR-103 and miR-744 were significantly downregulated in 95D/MTA1-si1# cells, with 0.290, 0.466, 0.431, 0.498, and 0.496-fold decreases, respectively, compared with 95D/NC cells.

In order to exclude possibilities of false-positive results coming from miRNA microarray analysis, we performed quantitative RT-PCR analysis to verify the expression of miR-125b, miR-210, miR-500, miR-194, miR-103 and miR-744. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) results revealed that miR-125b was upregulated, while miR-210, miR-500, miR-194 and miR-103 were downregulated in the 95D/MTA1-si1# cells, which was in accordance with the results from the miRNA microarray (Fig. 4B).

Discussion

As outlined in the introduction, the deregulation of MTA1 has been found in various human malignancies. Our previous study also found that MTA1 overexpression was significantly correlated with tumor progression and poor prognosis of NSCLC (7). However, the exact function of MTA1 in the tumor cell biology of lung cancer has not been thoroughly investigated. To address this issue, we evaluated MTA1 expression with regard to possible direct correlations with cell growth and invasion of lung cancer cells.

The functions of MTA1 on tumorigenesis and cell cycle progression are still unclear. In the present study, our data did not reveal that the silencing of MTA1 had any detectable effect on proliferation, colony formation, or cell cycle progression of 95D cells. This observation is in contrast to results of the study by Jiang *et al* (14). However, the divergent findings about the effect of MTA1 on tumor growth and cell cycle progression have been previously reported (15-18). While the reasons of this apparent discrepancy are currently unknown, we presumed that MTA1 could function as an activator or repressor on cell growth in a cellular context-dependent manner through influencing the balance between different genes relating to growth.

Tumor metastasis consists of multiple sequential steps regulated by various genes. The breakdown of the base membrane is considered as the initial and one of the most characteristic steps during the cascade of metastasis. In this study, downregulation of MTA1 in 95D cells by MTA1 gene silencing showed significantly decreased migration and invasion. These *in vitro* studies provide evidence that MTA1 may play important roles in lung cancer development. Our results are consistent with the *in vitro* studies on esophageal carcinoma cells and melanoma cells performed by Qian *et al* (19,20). MTA1 downregulation greatly reversed the malignant phenotypes of cancer cells.

However, to date, the molecular mechanisms by which MTA1 enhances the metastatic potential of tumor cells are still poorly understood. Only a few reports suggest that MTA1

may promote tumor progression by participating in chromatin remodeling (21,22), hypoxia signaling pathway (23), or the epithelial-mesenchymal transition pathway (24).

miRNAs are small non-coding RNAs and have been implicated in the pathology of various diseases, including lung cancer. Both MTA1 and miRNAs play a regulatory role in the metastatic process of lung cancer. We thus sought to determine whether MTA1 is involved in lung cancer metastasis through regulating miRNA expression in lung cancer cells.

In this study, we detected that a number of miRNAs were altered upon MTA1 knockdown by employing miRNA microarray. Among the miRNAs with a fold change >2, we found that miR-125b was upregulated by MTA1 knockdown. It has been shown that miR-125b functions as a tumor suppressor in lung cancer (25), oral squamous cell carcinoma (26), prostate cancer (27) and bladder cancer (28). miR-125b was significantly correlated with good prognosis of liver cancer (29). Transfecting oral squamous cell carcinoma cells with exogenous miR-125b significantly reduced cell proliferation (26). miR-125b was found to be underexpressed in metastatic breast cancer and metastatic liver cancer compared with their primary tumors (30,31).

On the other hand, miR-210, miR-194, miR-500 and miR-103 were found to be downregulated by MTA1 knockdown. According to the literature, miR-210 is involved in hypoxia signaling pathway and has been correlated with poor prognosis of breast cancer and pancreatic cancer (32,33). miR-500 was found to be abundantly expressed in several human liver cancer cell lines, 45% of human hepatocellular carcinoma (HCC) tissue, and the sera of HCC patients (34). Furthermore, the presence of miR-500 has been associated with triple-negative breast cancer (35). High expression of miR-103 has been correlated with poor survival of esophageal cancer (36). However, the role of miR-194 in tumorigenesis is controversial. It was found to be upregulated in esophageal adenocarcinoma (37) and downregulated in squamous cell carcinoma of the tongue (38). A noticeable phenomenon is that miR-192 was verified to enhance colony suppression and cell cycle arrest, partially dependent on the presence of wild-type p53 (39). This may partially support our previous hypothesis that MTA1 could influence the balance between different genes relating to growth.

In summary, silencing of MTA1 can effectively inhibit cell invasion and migration, and greatly reversed the malignant phenotypes of lung cancer cells. Furthermore, MTA1 can regulate the expression of some miRNAs which have a well-characterized association with cancer progression, suggesting that MTA1 could be involved in lung cancer metastasis through regulating miRNA expression in lung cancer cells. Although this study is the first to uncover the silencing of MTA1 as an inhibitor for cell invasion and migration via regulating miRNA expression, the complicated interaction between MTA1 and the miRNAs contributing to lung cancer is worthy of further investigation.

Acknowledgements

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