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Transforming growth factor-β1 induces epithelial-mesenchymal transition and increased expression of matrix metalloproteinase-16 via miR-200b downregulation in bladder cancer cells

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Abstract. Transforming growth factor-β1 (TGF-β1) is involved in the migration and metastases of bladder cancer. The present study was designed to investigate whether TGF-β1 is able to induce epithelial-mesenchymal transition (EMT) and the upregulation of matrix metalloproteinase-16 (MMP-16), and to identify an association between EMT and MMP-16 in bladder cancer. Following TGF-β1 treatment, samples of HTB9 and T24 bladder cancer cells were collected at various time points. Western blotting and quantitative polymerase chain reaction (qPCR) confirmed that TGF-β1 induced EMT in HTB9 and T24 cells at the protein and mRNA levels. The expression levels of the miR-200 family were determined by qPCR, which indicated that TGF-β1 treatment significantly reduced the expression of miR-200b. Bioinformatic analysis indicated that MMP-16 may be the target of miR-200b. Reporter luciferase assay confirmed that MMP-16 is a direct downstream functional target of miR-200b. A Matrigel migration assay demonstrated that miR-200b overexpression inhibited the migration of bladder cancer cells. In summary, the current study demonstrated that exogenous TGF-β1 leads to the induction of EMT and the downregulation of miR-200b in bladder cancer cells. To the best of our knowledge, this is the first evidence that MMP-16 is a direct target of miR-200b.

Introduction

Bladder cancer is the sixth most common type of cancer in the US, with 72,570 diagnoses estimated in 2013 (1). Patients with locally advanced or metastatic bladder cancer exhibit poor 5-year overall survival rates of 10-15% (2). The mechanisms of progression from superficial to muscle-invasive, and then to metastatic bladder cancer remain largely unknown. The activation of an epithelial-mesenchymal transition (EMT) program has been proposed as the critical mechanism for the acquisition of the invasive phenotype and the subsequent systemic spread of cancer cells. The loss of epithelial markers and the acquisition of mesenchymal morphological features are characterized by the disassembly of tight junctions and loss of normal cellular polarity due to decreasing expression of E-cadherin and increasing expression of various mesenchymal markers, including vimentin and N-cadherin (3). It is widely accepted that EMT is altered in various types of tumor, including bladder cancer (4).

Transforming growth factor-β1 (TGF-β1) controls cell proliferation and differentiation during embryonic development (3), and numerous studies have indicated that it is also associated with migration and metastases in multiple types of malignant tumor (5-7). TGF-β1 has been demonstrated to induce the expression of matrix metalloproteinases (MMPs), which facilitate tumor cell invasion and metastasis via the degradation of extracellular matrix. MMP-16 is a membrane-anchored MMP that is able to activate other MMPs (MMP-2 and 9), growth factors and receptors, and thereby facilitates a local cellular mechanism for migration (8). TGF-β signaling also regulates pathological EMT, inducing a number of diseases ranging from inflammatory disorders to fibrosis and cancer (9).

microRNAs (miRNAs) are short, single-stranded RNA molecules that interact with the 3' untranslated region (UTR) of mRNAs to regulate gene expression (10). An increasing number of studies have documented a link between the expression of miRNA and cancer pathogenesis. Several independent studies have indicated that the miR-200 family (miR-200a, 200b, 200c, 141 and 429) are downregulated in aggressive human tumors, and are potent inhibitors of EMT, tumor invasion and metastasis (4,11,12).

Therefore, the present study hypothesized that TGF-β1 may be involved in the regulation of EMT and the expression of MMP-16 in bladder cancer. The present study was designed to investigate whether TGF-β1 is able to induce EMT

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and upregulation of MMP-16, and to identify an association between EMT and MMP-16 in bladder cancer.

Materials and methods

Cell culture and reagents. The human bladder cancer cell lines, HTB9 and T24 (American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 3.75% fetal bovine serum (Gemini Bio-Products, Woodland, CA, USA) and 100 U/ml streptomycin-penicillin sulfate (Life Technologies, Grand Island, NY, USA) at 37˚C in a humidified atmosphere with 5% CO_2. Recombinant human TGF-β1 was obtained from R&D Systems (Minneapolis, MN, USA) and used at the specified concentrations. TGF-β1 treatment was conducted without serum deprivation.

Semi-quantitative polymerase chain reaction (qPCR). Total RNA from cell samples was extracted using the RNeasy Mini kit (Qiagen, Alameda, CA, USA). Complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The cDNA was synthesized from 2 μg total RNA on a PTC-200 Peltier Thermal Cycler DNA engine (MJ Research Inc., Waltham, MA, USA). The thermal cycler was used with a Chromo 4™ Real-Time Detection system and Opticon Monitor software, version 3.1 (Bio-Rad, Hercules, CA, USA) for qPCR analysis. Cycle threshold (Ct) values were normalized to the housekeeper GAPDH gene, and comparative quantification was performed based on a $2^{-\Delta\Delta Ct}$ calculation method (13). The specific primer sequences were as follows: GAPDH F, 5'-ACCACAGTCCATGCCATCAC-3' and R, 5'-TCCACCACC CTGTTGCTGTA-3'; N-cadherin F, 5'-AACCCTTATTTTGCC CCCAAT-3' and R, 5'-TCAACATGGTACCGGCATGA-3'; E-cadherin F, 5'-CGGGAATGCAGTTGAGGATC-3' and R, 5'-AGGATGGTGTAAGCGATGGC-3'; vimentin F, 5'-GAC CTCTACGAGGAGGAGAT-3' and R, 5'-TTGTCAACATCCTG TCTGAA-3'.

For mature miRNA analysis, total RNAs were extracted from cell cultures with a mirVana™ miRNA Isolation kit according to the instructions of the manufacturer (Applied Biosystems). For miRNA detection, qPCR was performed using TaqMan miRNA assays (Applied Biosystems) with specific commercial primer sets. All reagents and protocols were from Applied Biosystems, and detection was performed using U6 as an internal control. miRNA-specific qPCR was performed in triplicate and repeated three times.

Protein characterization. For western blot assessment, the cells were plated in culture dishes. Following treatment, the untreated control and TGF-β1-treated cells were harvested by scraping and then washed with phosphate-buffered saline (PBS). Cells were collected following centrifugation at 856 x g and pellets were resuspended in lysis solution (10 mM Tris, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.5% NP-40, 0.3 mM PMSF, 10 μg/ml Aprotinin). Protein estimation was performed with crystal violet protein dye (Sigma-Aldrich) using a DU 800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). Protein (50 μg) was electrophoresed using NuPAGE 4-12% Bis-Tris gel (Invitrogen Life Technologies, Carlsbad, CA, USA). The protein was transferred to a nitrocellulose membrane (Invitrogen Life Technologies) using a wet method at 100 volts for 1 h. The membrane was then blocked with 5% (w/v) milk and placed in a rotator for 1 h at room temperature. The primary antibody was added to the culture with milk (2.5% w/v) and allowed to incubate overnight at 4˚C. The membrane was then washed with PBS/0.05% Tween-20 three times (15 min each) prior to the addition of the appropriate horseradish peroxidase-linked secondary antibody and incubation for 1 h at room temperature. The primary antibodies were anti-E-cadherin, anti-vimentin (Cell Signaling Technology, Danvers, MA, USA), anti-MMP-16 (Chemicon, Temecula, CA, USA), anti-N-cadherin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and anti-GAPDH (Santa Cruz Biotechnology, Inc.). The membrane was then washed three times for 15 min each, prior to the addition of SuperSignal Chemiluminescent substrate (Pierce Biotechnology, Inc., Rockford, IL, USA) and then immediately visualized using a ChemiDoc Imaging system (Bio-Rad).

Plasmid construction and dual-luciferase reporter assay. To validate whether miR-200b was able to suppress the expression of MMP-16 by directly targeting the 3'UTR region, luciferase assays were performed. Changsha Yinrun Biotechnologies Inc. (Changsha, China) constructed pGL3-MMP-16 by amplifying the 3'UTR of the MMP-16 gene harboring the miR-200b binding site, as predicted by TargetScan (http://www.targetscan.org), and subsequently cloning it into the pGL3 control vector (Promega, Madison, WI, USA) at the *Xba*I site immediately downstream of firefly luciferase (f-luc). pGL3-MMP-16-mut, which contained deletions of the MMP-16 3'UTR target regions, was generated to be used as a negative control. For the luciferase assay, HTB9 and T24 cells were cultured in 12-well plates and each cotransfected with 400 ng of either pGL3-MMP-16 or pGL3-MMP-16-mut, 50 ng pRL-TK (Promega) and 50 nmol/l 200b mimics or negative control (NC). Cells were lysed after 48 h, and luciferase activity was determined using the Dual-Luciferase Reporter Assay system (Promega). The results were expressed as relative luciferase activity (firefly luciferase/Renilla luciferase).

Transwell assay. Cells were transfected with control or miR-200b mimic for 48 h and then were treated with or without TGF-β1 (10 ng/ml) for 24 h. $5x10^4$ cells were resuspended and added to the upper chamber of a Transwell system (BD Biosciences, Franklin Lakes, NJ, USA). Cells were incubated for 24 h, and those that did not migrate through the pores were removed by scraping the upper surface of the membrane with a cotton swab. Cells that had migrated to the lower surface of the membrane were fixed for 5 min in 100% methanol (Sigma-Aldrich) and stained with 0.2% crystal violet. The cells that migrated through the insert were counted in five random fields and expressed as the average number of cells/field. All experiments were conducted in triplicate and performed at least three times.

Statistical analysis. Data were analyzed using SPSS software, version 11.0 (SPSS Inc., Chicago, IL, USA) for Windows. $P<0.05$ was considered to indicate a statistically significant difference.

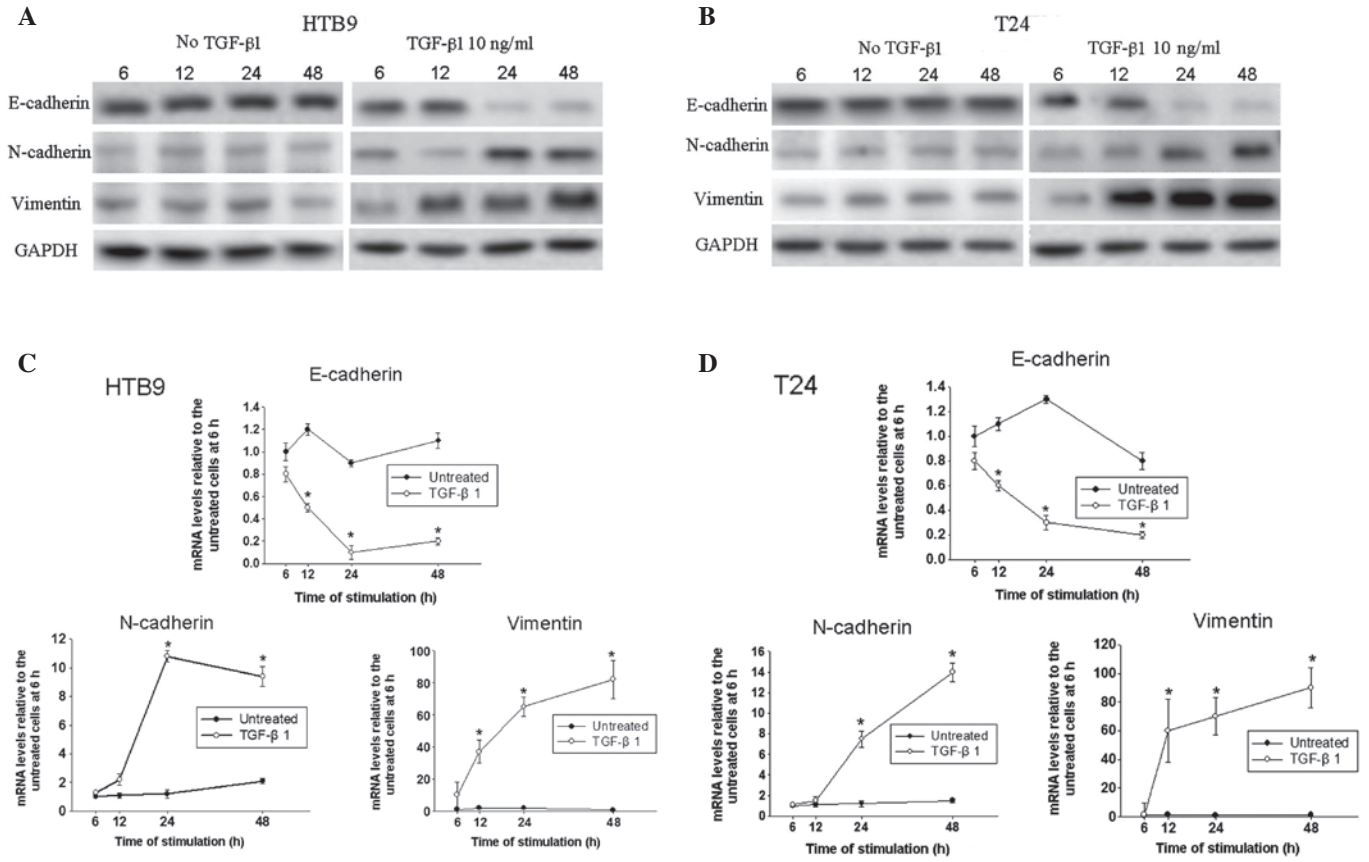

Figure 1. Time-course western blots and qPCR experiments were used to study the changes of EMT markers at the protein and mRNA levels in (A and C) HTB9 and (B and D) T24 cells. The concentration of TGF-β1 was 10 ng/ml. Graphs C and D display the pooled data (the mean ± standard error) from three independent experiments. *P<0.05 vs. control cells. TGF-β1, transforming growth factor-β1; qPCR, quantitative polymerase chain reaction; EMT, epithelial-mesenchymal transition.

Results

TGF-β1 induces EMT in HTB9 and T24 bladder cancer cell lines. EMT is associated with progression and poor prognosis in numerous types of cancer, and EMT can be induced by TGF-β1 (14,15). In the present study, a time-course western blot analysis was designed to investigate the expression of EMT markers, including E-cadherin, N-cadherin and vimentin. The expression of all these markers remained stable at the protein level in the untreated HTB9 and T24 cells throughout the 48 h (Fig. 1A and B). However, following TGF-β1 treatment of HTB9 and T24 cells, the expression of E-cadherin was progressively downregulated in a time-dependent manner, while the expression levels of N-cadherin and vimentin were increased, particularly after 24 h. qPCR was used to confirm whether mRNA levels were altered in a manner consistent with the changes observed at the protein level. A reduction in E-cadherin mRNA levels in the TGF-β1-treated cells compared with those in control cells was first observed after 6 h of treatment (Fig. 1C and D). N-cadherin and vimentin mRNA expression levels were significantly upregulated following TGF-β1 treatment, which is consistent with the observations from the western blots.

TGF-β1 reduces the expression of miR-200b. The miR-200 family is considered to be essential in EMT. Therefore, changes in expression levels of these miRNAs in untreated and TGF-β1-treated bladder cancer cells were analyzed at different time points in the current study (Fig. 2). There were no significant differences in the expression levels of miR-200a, 429 and 200c between untreated and treated cells (Fig. 2A, C and E). No significant difference in levels of miR-141 expression between untreated and treated cells was detected until 48 h after TGF-β1-treatment (Fig. 2D). However, expression levels of miR-200b were significantly downregulated from 12 h post-TGF-β1 treatment (Fig. 2B). TGF-β1 reduced the expression levels of miR-200b progressively in a time-dependent manner in HTB9 and T24 cells. This suggests that miR-200b is a key factor in TGF-β1-induced EMT.

Expression of MMP-16 in HTB9 and T24 cells increases following TGF-β1 treatment. Bioinformatic analysis indicated that MMP-16 may be the target of miR-200b; positions 1590-1595 of the MMP-16 3'UTR are the interaction sites of miR-200b, as predicted by TargetScan (Fig. 3A). Therefore, the expression of MMP-16 in the two cell lines was examined, and the western blots implied that its expression was increased by TGF-β1 in a time-dependent manner in the two cell types (Fig. 3B).

MMP-16 is a direct downstream functional target of miR-200b. To validate the direct binding of miR-200b to the MMP-16

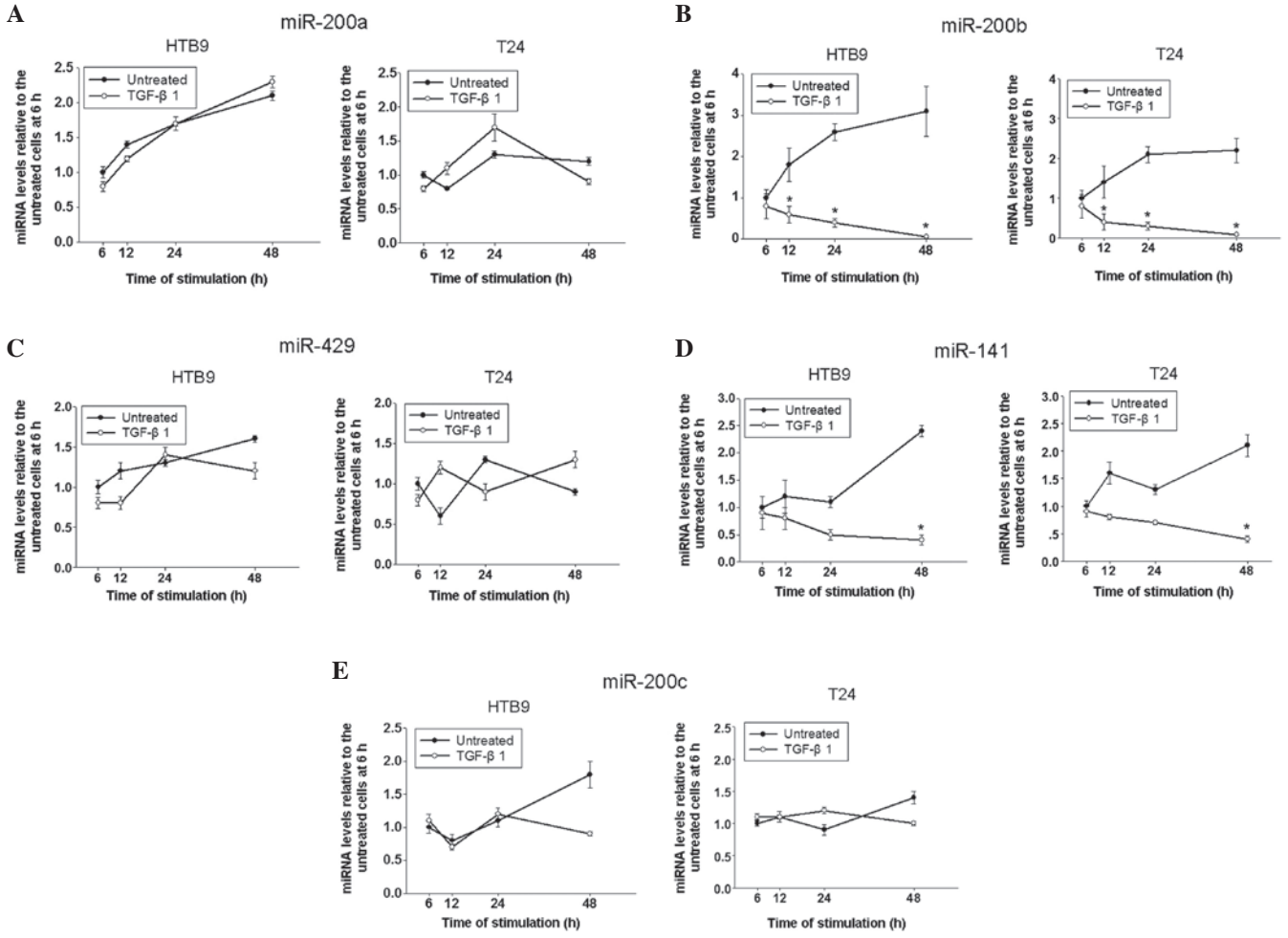

Figure 2. Expression levels of five miR-200 family members in TGF-β1-treated and untreated HTB9 and T24 cells were determined by qPCR. (A) miR-200a; (B) miR-200b; (C) miR-429; (D) miE-141; and (E) miR-200c. The concentration of TGF-β1 was 10 ng/ml. The results represent the mean ± standard error of three independent experiments. *P<0.05 vs. untreated control cells. qPCR, quantitative polymerase chain reaction; TGF-β1, transforming growth factor-β1.

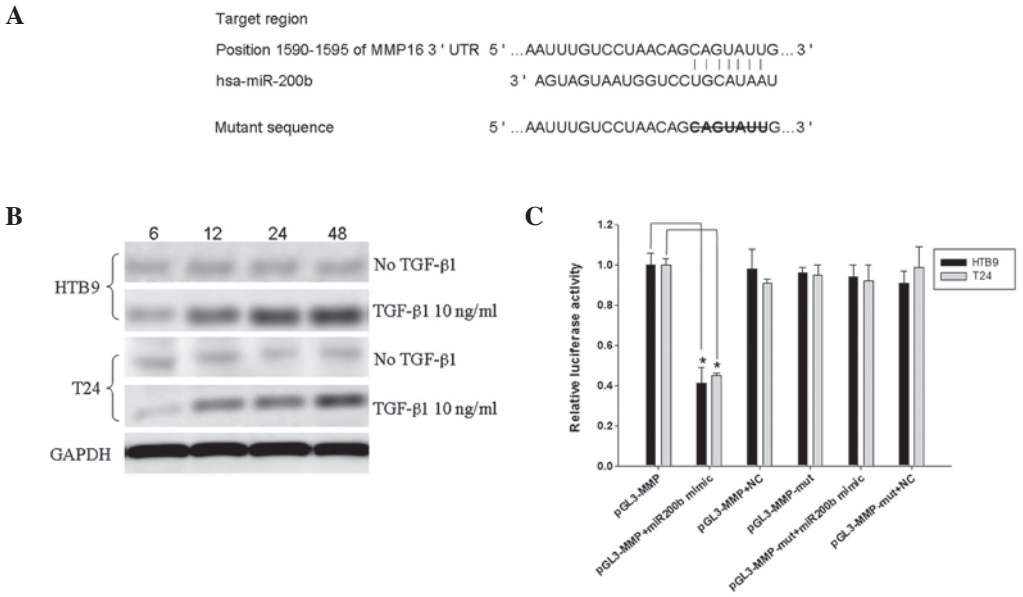

Figure 3. MMP-16 serves as a novel direct target of miR-200b. (A) Bioinformatic analysis revealed a potential miR-200b binding site at the 3'UTR of MMP-16, as predicted by TargetScan. Mutated sequences used are displayed in the figure. (B) Western blots displaying MMP-16 expression at the time points 6, 12, 24 and 48 h. (C) Luciferase assay confirming that MMP-16 was a direct target of miR-200. HTB9 or T24 cells were cotransfected with miR-200b mimics or NC and pGL3-MMP-16 or pGL3-MMP-16-mut vector. Firefly luciferase activity is normalized to Renilla luciferase activity. The data are presented as the mean ± standard error of three independent experiments (*P<0.05). TGF-β1, transforming growth factor-β1; MMP, matrix metalloproteinase; UTR, untranslated region; NC, negative control.

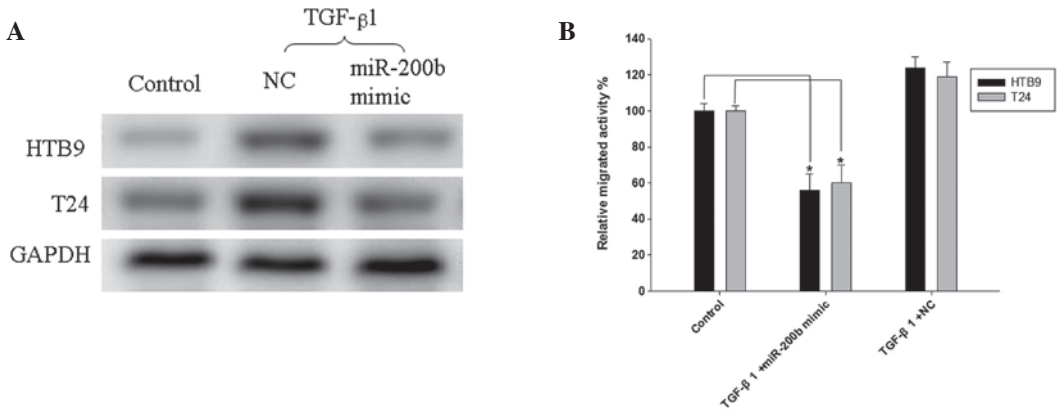

Figure 4. TGF-β1-induced MMP-16 upregulation and invasive ability were abrogated by miR-200b mimic transfection in HTB9 and T24 cells. The cells were treated with or without TGF-β1 (10 ng/ml) for 24 h following transfection with miR-200b mimic or NC for 48 h. (A) The expression of MMP-16 was determined by western blot analysis. (B) Decreased Matrigel invasion with HTB9 and T24 cells was observed with miR-200b mimic transfection. Quantitative results for the transmembrane ability of cells were as shown. *P<0.05. TGF, transforming growth factor-β1; NC; negative control; MMP, matrix metalloproteinase.

3' UTR region, an miR target reporter luciferase assay was performed in HTB9 and T24 cells. Levels of luciferase activity in HTB9 or T24 cells cotransfected with miR-200b mimics and the pGL3-MMP-16 vector were reduced compared with levels in controls (Fig. 3C), indicating that MMP-16 is one of the functional downstream targets of miR-200b.

miR-200b overexpression inhibits expression of MMP-16 and the migration of bladder cancer cells. The expression levels of MMP-16 were measured, in view of the changes of miR-200b expression levels in HTB9 and T24 cells induced by miR-200b mimic or NC transfection (Fig. 4A). The expression of MMP-16 was downregulated following miR-200b mimic transfection compared with that following NC transfection. A Matrigel migration assay demonstrated that TGF-β1 slightly enhanced the migration of HTB9 and T24 cells transfected with NC compared with that of non-treated control cells (Fig. 4B). However, miR-200b mimic transfection significantly inhibited the migration in the two cell lines when treated with TGF-β1 (Fig. 4B).

Discussion

In the present study, the results indicated that exogenous TGF-β1 leads to reduced expression of E-cadherin and downregulated levels of miR-200b in the bladder cancer cell lines, HTB9 and T24. Bioinformatic analysis demonstrated that MMP-16 may be a novel downstream target of miR-200b, and this was confirmed in two cell lines. Further experiments indicated that miR-200b overexpression inhibited TGF-β1-induced MMP-16 upregulation and the migration of bladder cancer cells. The results of the present study indicated that TGF-β1 downregulated the levels of miR-200b and increased those of MMP-16. This may be a novel mechanism of invasion and metastasis in bladder cancer.

TGF-β1 is a cytokine that enhances cell characteristics associated with invasiveness, metastasis and motility (14,16). EMT is associated with progression and poor prognosis in various types of cancer, and can be induced by TGF-β1 (14). In a study by Koo *et al* (15), AY-27 rat bladder cancer cells were incubated with TGF-β1 and were observed to have reduced E-cadherin and increased vimentin immunoreactivity. Their results were consistent with results observed in the present study.

Gregory *et al* (17) treated Madin-Darby canine kidney epithelial cells with TGF-β1 and noted that all the members of the miR-200 family were selectively downregulated, indicating that the miR-200 family is involved in TGF-β1-induced EMT. Slabáková *et al* (14) and Starsíchová *et al* (18) described that BPH-1 benign prostate hyperplasia cells underwent EMT following TGF-β1 treatment, but also that there were no apparent changes in the expression levels of the miR-200 family members following TGF-β1 treatment. They hypothesized that the expression of the miR-200 family members was not regulated by TGF-β1 at the onset of the EMT process, but may oppose the reversal of the EMT phenotype. In the present study, only miR-200b was significantly downregulated following TGF-β1 treatment in two bladder cancer cell lines. It was hypothesized that miR-200b may be a key member in the TGF-β1-induced EMT process of bladder cancer. The discrepancy between results of the current study and those of previous studies may be due to the use of different cell lines in the experiments. The previous cell lines were derived from benign tissue, while the cells used in the current study were malignant.

The miR-200 family is a cluster of miRNAs that are positioned at two different loci in the genome (19). Two studies by Hurteau *et al* (20,21) revealed that one individual member of the miR-200 family, miR-200c, was able to simulate the effects of the function of the cluster and be sufficient to regulate ZEB1 and restore E-cadherin in breast cancer cells. Loss of expression of the miR-200 family members may be critical in the repression of E-cadherin during EMT, thereby enhancing migration and invasion during cancer progression (22). In the present study, the results suggested that TGF-β1 treatment led to reduced expression levels of E-cadherin and one individual member, miR-200b.

The enzymatic activity of MMPs is tightly controlled at multiple levels. MMP-16 can not only directly degrade certain matrix molecules, but can also activate other MMPs (23). miR-15 and miR-146-5p have been demonstrated to induce MMP-16 to inhibit migration and invasion of glioma and cardiomyocyte progenitor cells (8,23,24). To the best of our

knowledge, the current study is the first to provide evidence that miR-200b targeted the MMP-16 3' UTR regions via reporter luciferase assay in two bladder cancer cell lines. Additionally, the upregulation approach was employed to investigate the role of miR-200b in the invasive ability of bladder cancer cells. miR-200b inhibited cell migration, establishing that miR-200b serves as a metastasis-inhibiting miRNA in bladder cancer.

A limitation of the present study was that the mechanism of the TGF-β1-induced repression of miR-200b was not assessed. DNA hypermethylation has previously been associated with the silencing of miR-200 family members in muscle-invasive bladder tumors and poorly differentiated bladder cell lines (4). It was therefore hypothesized that TGF-β1 mediates the epigenetic silencing of miR-200 family members, which requires further investigation.

In summary, exogenous TGF-β1 led to the induction of EMT and the downregulation of miR-200b in bladder cancer cells. To the best of our knowledge, the current study provided evidence that MMP-16 is a direct target of miR-200b for the first time.

Acknowledgements

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