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MicroRNA-101 suppresses motility of bladder cancer cells by targeting *c-Met*

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ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNAs that play regulatory roles by repressing translation or cleaving RNA transcripts. Here, we report that the expression of microRNA-101 (miR-101) is down-regulated in human bladder cancer tissue versus normal adjacent tissue. To better characterize the role of miR-101 in bladder cancer, we conducted a gain-of-function analysis by transfecting the bladder cancer cell line T24 with chemically synthesized miR-101 mimics. We found that miR-101 directly targets *c-Met* via its 3′-UTR. Specifically, forced expression of miR-101 decreased *c-Met* expression at both mRNA and protein levels, consequently inhibiting T24 cell migration and invasion in a *c-Met*-dependent manner. In conclusion, we have shown miR-101 to be a novel suppressor of T24 cell migration and invasion through its negative regulation of *c-Met*.

1. Introduction

Urinary bladder cancer ranks ninth in worldwide cancer incidence [1]; the worldwide age-standardized rate (ASR) for this disease is 10.1 per 100,000 for men and 2.5 per 100,000 for women [2]. The USA has an estimated 73,510 new cases, with 14,880 cases arising in 2012 [3]. An improved understanding of the molecular mechanisms that drive urothelial transformation and the growth, invasion, and metastasis of bladder cancer has been gained from studies reporting the dysregulation of several relevant protein-coding genes and their associated signaling pathways, including HGF/*c-Met* [4], WNT/beta-Catenin [5], and insulin-like growth factor [6]. However, the roles of miRNAs in bladder cancer and their potential mechanisms of action are largely unexplored.

MiRNAs are small (~22 nucleotides) non-coding RNAs that suppress gene expression by interacting with the 3′-untranslated regions (3′-UTRs) of target mRNAs. These interactions may result in either inhibited translation or degradation of the target mRNAs [7]. Substantial evidence supports a functional role for miRNAs in different cancers; ~50% of miRNAs are located at fragile sites in the genome which are commonly amplified or deleted in human cancers [8]. Furthermore, mis-expression of miRNAs has been observed in various types of cancer and is also correlated with the clinical outcome of cancer patients. Ample evidence indicates that miRNAs may regulate tumorigenesis by functioning as either oncogenes or tumor suppressors [9]. Several studies found that miRNAs regulate cell migration and invasion in diverse carcinomas [10–12]. Cell migration and invasion are normal physiological processes that occur throughout embryonic development [13]. However, pathological migration and invasion are associated with metastasis [14]. Most prior studies on miR-101 have indicated that it is significantly under-expressed in multiple types of cancer, including in lung [15], prostate [16], gastric [17], liver [18] and colorectal cancer [19], and displays a suppressive effect on cellular migration and invasion.

In this study, we demonstrated that miR-101 regulated *c-Met* expression in T24 cells and that forced expression of miR-101 led to reduced cell motility and invasive capacity, deficits which could be partially rescued by forced *c-Met* expression.

2. Materials and methods

2.1. Reagents

The miR-101 mimic (designated miR-101, sense; 5′-UACAGUACUGUGAUAACUGAA-3′) and a negative control duplex lacking any significant homology to known human sequences (designated NC, sense; 5′-ACUACUGAGUGACAGUAGA-3′) were used in a transient gain-of-function study. A small interfering RNA targeting nucleotides 512–531 of the human *c-Met* mRNA (Genbank accession number NM_001127500) was designed as described before (designated siMet, sense; 5′-GGAGGUGUUUGGAAAGAUA-3′) [20]. All RNA duplexes were chemically synthesized by GenePharma (Shanghai, China). The primary immunoblotting antibodies used in the experiments were anti-Met and anti-GAPDH (Epitomics, Burlingame, CA).

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2.2. Tissue samples

Tissue samples from bladder cancer and the adjacent normal urothelium were collected from patients undergoing radical cystectomy for bladder cancer in the Department of Urology at the First Affiliated Hospital of Zhejiang University in China. The surgeries occurred between January and April 2011, and all patients provided signed informed consent. Immediately following resection, the samples were placed in liquid nitrogen storage until the time of RNA extraction.

2.3. Cell culture and transfection

The human bladder cancer cell line T24 (Shanghai Institute of Cell Biology, Chinese Academy of Sciences) was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 μg/ml streptomycin and 50 U/ml penicillin in a humidified atmosphere with 5% CO_2 at 37 °C. The day before transfection, cells were plated at 60–70% confluency in medium without antibiotics. Transfections were performed using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.4. RNA isolation and real-time PCR

The miR-101 and *c-Met* expression levels were measured using quantitative real-time RT-PCR. MicroRNAs were extracted from human tissues or cancer cell lines using the RNAiso for small RNA kit (Takara, Dalian, China). Prior to detection, miRNA was poly-adenylated and reverse transcribed into cDNA using the One Step PrimeScript miRNA cDNA Synthesis Kit (Takara, Dalian, China). Total RNA was extracted using TRIzol reagent (Takara, Dalian, China) and reverse transcribed into cDNA using the PrimeScript RT reagent Kit (Takara, Dalian, China). The resulting cDNAs were quantified using SYBR Green (Takara, Dalian, China) with an ABI 7500 fast real-time PCR System (Applied Biosystems, Carlsbad, USA). The small nuclear RNA U6 and GAPDH mRNA were used as internal controls for miRNA and mRNA detection, respectively. The qPCR primers, obtained from Sango Biotech (Shanghai, China), had the following sequences: mir-101 (forward: 5′-TACAGTAC TGTGATAACTGAA-3′), U6 (forward: 5′-TGCGGGTGCTCGCTTCGG-CAGC-3′), *c-Met* (forward: 5′-TGTCCCGAGAATGGTCATAA-3′; reverse: 5′-AGGGAAGGAGTGGTACAACA-3′) and GAPDH (forward: 5′-AAGGTGAAGGTCGGAGTCA-3′; reverse: 5′-GGAAGATGGTGATGG GATTT-3′).

2.5. Wound healing assay

Approximately 1×10^5 T24 cells were plated in each well of a 6-well plate. After overnight incubation, the cells were treated with dsRNAs (50 nM NC, 50 nM miR-101 mimics, or 50 nM siMet) for 72 h, after which time the cells reached full confluency. The cell monolayers were wounded by scraping them with a micropipette tip, washed several times with media to remove dislodged cells, and placed back in growth medium. Photographs were taken using a phase-contrast microscope (Olympus, Japan) immediately following or 24 h after wounding. Cell migration was evaulated with an inverted bright field microscopy under the 4× objective as described previously [21]. Only cell-free area was selected, measured, and quantified using Image ProPlus software and calculated as percentage cell migration using the equation: %cell migration = [1 − (final scratch area/initial scratch area)]. These experiments were repeated three times.

2.6. Cell migration and invasion assay

Cells were plated on a 6-well plate at a density of 8×10^4 cells/well. The cells were serum-starved for 24 h prior to treating them with dsRNAs (50 nM NC, 50 nM miR-101 mimics, or 50 nM siMet) and harvested 72 h following treatment. The transfected cells and corresponding control cells were suspended in medium at a concentration of 4×10^5 cells/ml, and 200 μl of each suspension was added to the top chamber of a well containing an uncoated (for motility assays) or Matrigel-coated (for invasion assays) PET membrane (24-well insert, 8-μm pore size; Millipore, Bedford, MA). Medium (600 μl) supplemented with 20% fetal bovine serum was added to the lower chamber of each well to act as a chemo-attractant. The cells were incubated for 24–48 h, and those cells 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 and stained for 2 min with 0.1% crystal violet. These invading cells were counted in five random fields, and their numbers were expressed as the average number of cells per field. The experiments included three replicates for each condition and were performed a minimum of three times. Five visual fields of 200× magnification of each insert were randomly selected and counted for the cell numbers under a light microscope.

2.7. Protein extraction and Western blotting

Forty-eight hours after transfection, the cells were lysed in cell lysis buffer (Cell Signaling). The total protein concentration in each lysate was determined using the BCA Protein Assay kit (Pierce), and equal amounts of total protein from each sample were separated on 10% SDS–polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked for 1 h with 1% BSA in Tris-buffered saline containing 0.05% Tween 20, incubated overnight with primary antibody, washed, and incubated with secondary antibody, and bands were visualized by chemi-luminescence. The antibodies used were anti-Met (Epitomics, Burlingame, CA) and anti-GAPDH (Cell Signaling Technology, Beverly, MA).

2.8. Luciferase assays

To construct luciferase reporter vectors, the *c-Met* 3′-UTR, which contains putative binding sites for miR-101, was amplified from genomic DNA using the met 3′UTR primers (forward: 5′-TCG**Agagctc**GGTTCCAATCACAGCTCATAGG-3′; reverse: 5′-TCG**Atct aga**GCAACAACAGCCAAACTCAAC-3′). The amplified fragment was inserted into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, USA) between the SacI and XbaI sites. In addition, a mutant 3′-UTR carrying a mutation in the miR-101 seed region was generated on a pMD-18 T vector backbone (TaKaRa, Japan) using met 3′UTR mutant primers (forward: 5′-TGCTTGC**ATGA-CAT**TAGTGCAT-3′; reverse: 5′-CAAAATTCCACAGAATGAAGCATCA C-3′) and the MutanBest Kit (TaKaRa, Japan) and then sub-cloned into pmirGLO Dual-Luciferase Vector. Both insertions were verified by sequencing. HEK 293T cells plated on a 24-well plate were co-transfected with 50 nM of either miR-101 mimics or NC oligos and with 200 ng reporter plasmid containing either wild-type or mutant 3′-UTR. The relative luciferase activity in each sample was measured using the Dual-Luciferase Reporter Assay System (Promega, USA) 48 h after transfection.

2.9. c-Met rescue experiments

The pT-Met plasmid was constructed by inserting the human *c-Met* coding sequence into the pTarget vector (GeneCopoeia). Cells

were co-transfected with miR-101 mimics or NC oligos and with pT-Met or the empty pTarget vector. The cells were collected 24 h after transfection and analyzed for migration and invasion activity as described above. The *c-Met* expression was verified by Western blotting.

2.10. Statistical analysis

Data were expressed as the mean ± standard deviation (SD) for three independent experiments. Differences between samples were analyzed by independent sample *t*-tests using SPSS V17.0 software. Correlation between two groups was analyzed using bivariate correlation analysis. Statistical significance was designated at $P < 0.05$ as compared with the corresponding control or as specifically indicated.

3. Results

3.1. Expression of mature miR-101 in bladder cancer tissues and the T24 cell line

To verify the expression of miR-101 in human bladder cancer tissues and in a bladder cancer cell line, we used real-time RT-PCR to quantify and compare miR-101 expression levels in 10 pairs of human bladder cancer tissues versus adjacent normal mucosal tissues and in T24 cells versus SV-HUC-1 cells (a normal transitional epithelial cell line). We found that miR-101 expression levels were generally decreased in cancerous tissues compared to their corresponding non-cancerous controls, with 7 out of 10 cancerous tissues exhibiting a 50% relative reduction in miR-101 (Fig. 1A). Analysis of the T24 cell line gave similar results (Fig. 1B). This finding was consistent with fold-change data from a previous study [22]. Thus, we speculated that miR-101 might be a tumor suppressor in bladder cancer.

3.2. miR-101 decreased the migration and invasive capacity of T24 cells

Previous studies demonstrated that miR-101 inhibits migration and invasion in gastric cancer and lung cancer cells [23,24]. We wondered whether miR-101 could regulate bladder cancer cells' capacity for migration and invasion. T24 cells were therefore transfected with miR-101 mimics or siMet before evaluating cell motility in both wound healing and transwell assays. Forced expression of miR-101 in T24 cells led to retarded wound closing compared to control oligonucleotide-transfected parental cells (Fig. 2A and C). Concordantly, forced expression of miR-101 in T24 cells significantly suppressed their migration and invasive activity (Fig. 2B and D). Thus, miR-101 is a negative regulator of migration and invasion in T24 cells.

3.3. miR-101 inhibited the expression of c-Met via binding to its 3′-UTR

Having demonstrated a functional role for miR-101 in T24 cells, we explored the cellular mechanisms underlying miR-101-dependent regulation of cell migration and invasion. TargetScan analysis (http://www.targetscan.org) was conducted in order to identify predicted miR-101 targets. Among the target genes identified, *c-Met* had significantly decreased expression on both the mRNA and protein levels in cells treated with miR-101 mimics (Fig. 3C and D). The target analysis identified a single predicted miR-101 binding site in the 3′ UTR of the *c-Met* mRNA (Fig. 3A); an alignment of miR-101 and the predicted miR-101 target site shows a conserved 7-bp "seed" sequence for the miR-101:mRNA pairing (Fig. 3A). To determine whether miR-101 specifically regulates

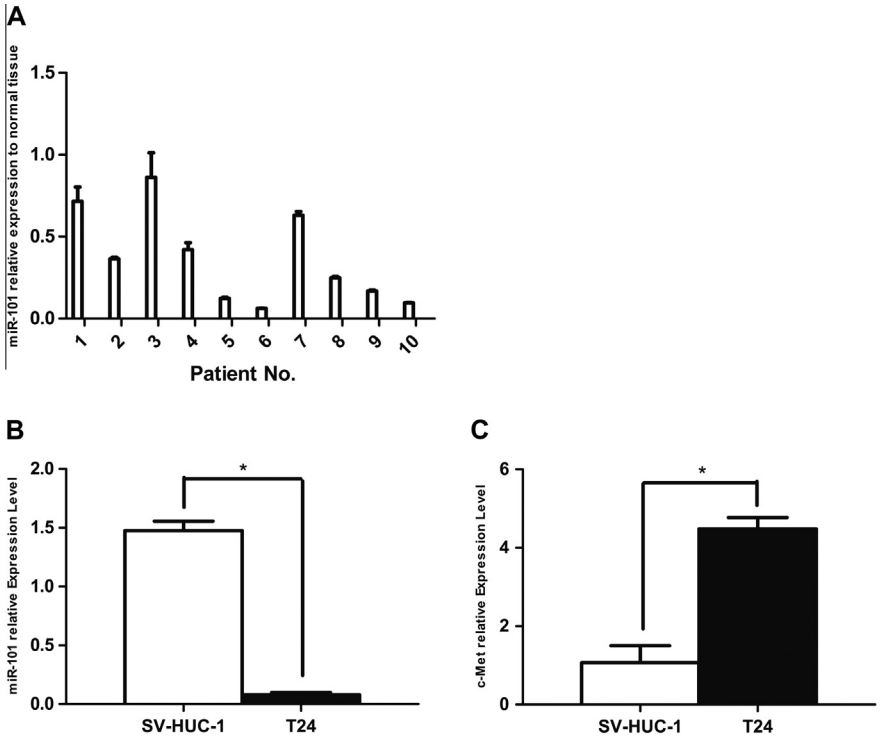

Fig. 1. MiR-101 expression is altered in human urinary bladder cancer samples and in the T24 cell line. Expression levels for miR-101 and *c-Met* obtained by real-time PCR analysis were normalized to U6 snRNA expression and to GAPDH expression, respectively. (A) The miR-101 levels in 10 bladder cancer tissue samples were normalized to the levels in patient-matched adjacent normal bladder tissue, which were assigned a value of 1.0. (B&C) Real-time PCR was used to detect and compare miR-101 and *c-Met* mRNAs in the T24 cell line versus SV-HUC-1 cells (*$P < 0.05$).

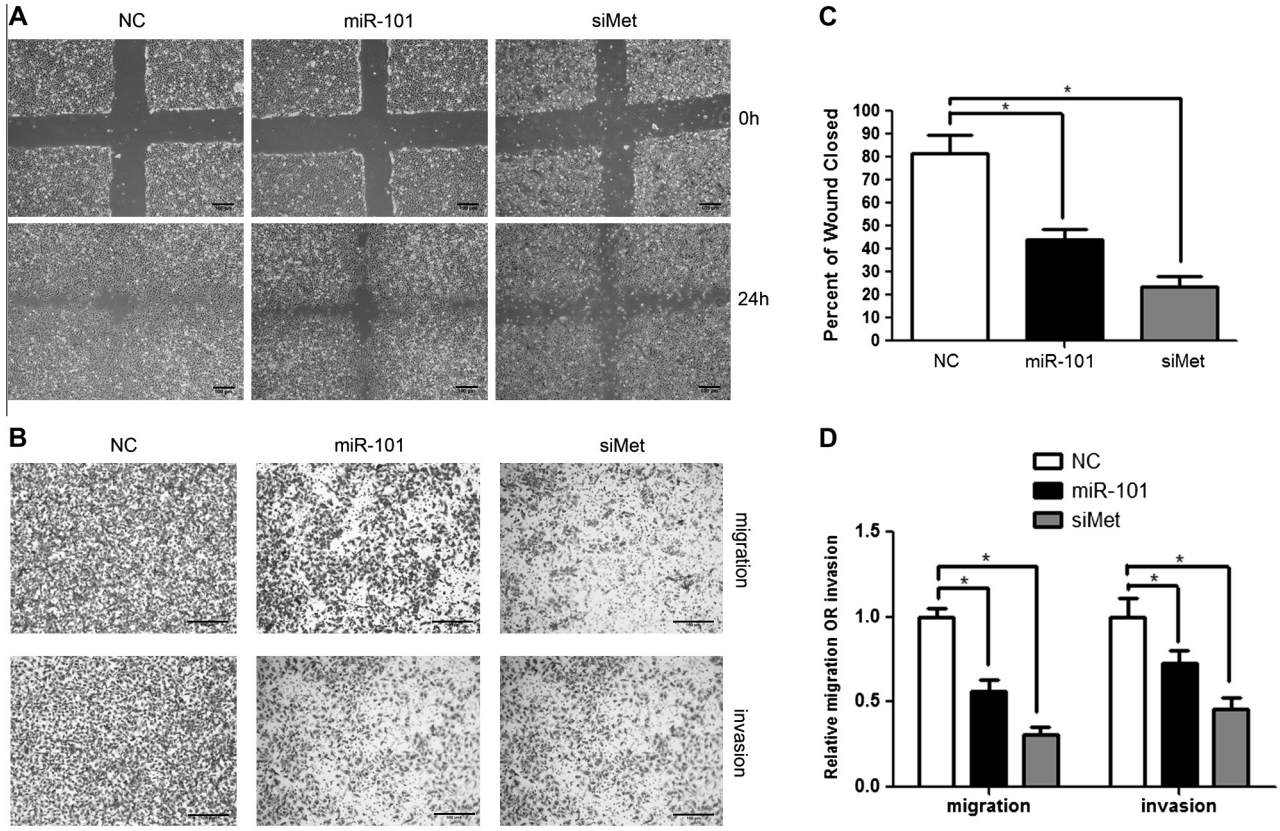

Fig. 2. Forced expression of miR-101 suppresses cell motility. (A) T24 cells were transfected with NC, miR-101 mimics or siMet and then used in a wound healing assay with a 24-h recovery period. (B) T24 cells were transfected with NC, miR-101 mimics or siMet, and cell migration and invasion were assessed after a 24-h incubation by transwell assay. (C) Quantification of cell motility by measuring the area between the invading front of cells in three independent experiments. The degree of motility is expressed as percent of wound closure as compared with the zero time point. (D) Representative migration and invasion results at 200× are shown in (B). Values are expressed as $\chi \pm s$ of 3 independent experiments performed in triplicate (*$P < 0.05$).

c-Met through the predicted binding site, we amplified the *c-Met* 3′-UTR sequence and inserted it down-stream of firefly luciferase in the pmirGLO Dual-Luciferase miRNA Target Expression Vector. A second vector with a mutation at the putative binding site was also constructed (Fig. 3A). As expected, forced expression of miR-101 in HEK 293T cells significantly reduced the activity of the luciferase reporter gene fused to the wild-type *c-Met* 3′-UTR by more than 50% (Fig. 3B). In contrast, the luciferase activity of the reporter carrying the mutated 3′-UTR was unaffected by co-transfection with miR-101. These data suggest that miR-101 directly binds to the 3′-UTR of *c-Met* to regulate its expression.

3.4. Restoration of c-Met expression rescues miR-101-suppressed cell motility

To determine the functional relevance of c-Met targeting by miR-101, we asked if forced *c-Met* expression could circumvent the inhibitory effects of miR-101 on cell motility. To this end, we inserted the human *c-Met* coding sequence into the pTarget vector and demonstrated that *c-Met* expression was restored after transfection of cells with this pT-Met construct (Fig. 4A). Cell migration and invasion were evaluated using the wound healing and transwell assays, respectively, following co-transfection of cells with either miR-101 mimics or NC oligos and with either pT-Met or the pTarget empty vector. We observed that the forced expression of *c-Met* rescued cell migration and invasion in the presence of miR-101 (Fig. 4B and C), showing that restored *c-Met* expression could reverse the miR-101-induced decrease in cell motility. Thus *c-Met* is a functionally relevant target downstream of miR-101 that mediates the migration and invasive capacity of T24 cells.

4. Discussion

Tumor metastasis and disease recurrence are the major causes of poor prognoses for bladder cancer patients. There is an urgent need to develop an effective targeted therapeutic strategy for improving survival outcomes for these patients [25]. Elucidating the molecular mechanisms of bladder cancer pathogenesis is critical to doing so. The role of miRNAs in cancer metastasis depends on their involvement in pathological cell migration and invasion, which are key functions in the biological processes of tumor metastasis [26]. Most studies have indicated that miR-101 is significantly under-expressed in various types of cancer, including gastric, prostate, breast, liver, and lung cancer [15,16,18,27,28]. Emerging evidence shows that miR-101 expression is lost in bladder cancer tissues and bladder cancer cell lines [22,29]. However, miR-101's functional role in bladder cancer is currently unclear. To the best of our knowledge, this study provides the first functional characterization and mechanistic examination of miR-101 in human bladder cancer cells. Our initial interest was in identifying the gene targets of miR-101 in bladder cancer cells, with a focus on the miRNA's role in cell migration and invasion. By utilizing a bioinformatics approach to search for potential miR-101 targets, we have identified *c-Met* as one of the pivotal targets.

We found *c-Met* to be a particularly interesting target for investigation due to its involvement in many types of cancer and its

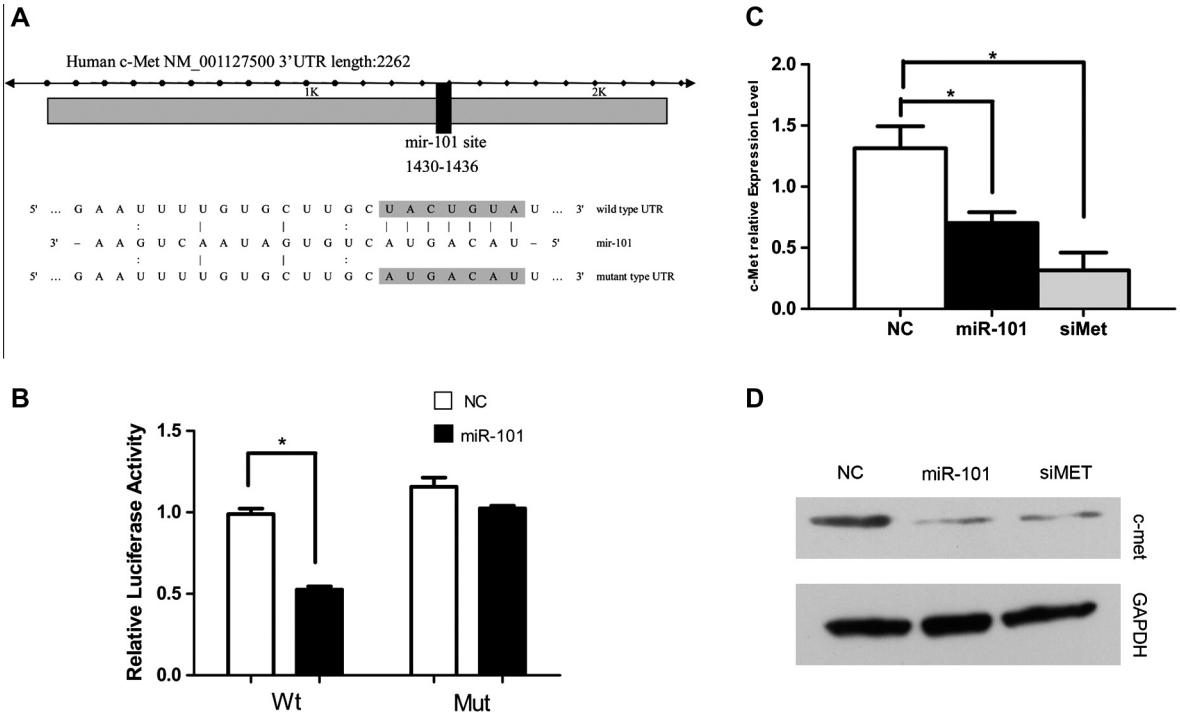

Fig. 3. MiR-101 directly targets *c-Met* by interacting with its 3′-UTR. (A) A predicted miR-101 binding site in the *c-Met* mRNA 3′-UTR is shown as a filled black rectangle (top). In an alignment between the predicted miR-101 target site and miR-101, the conserved 7-bp "seed" sequence for the miR-101:mRNA pairing is highlighted in grey (bottom). Mutations made to the seed sequence to generate the cMet Mut 3′-UTR are shown highlighted in grey below the seed region. (B) T24 cells were co-transfected with 50 nM of either miR-101 mimics or NC and 200 ng pmirGLO Dual-Luciferase miRNA Target Expression Vector containing either the Wt or Mut 3′-UTR of *c-Met*. Relative firefly luciferase activity normalized to renilla luciferase activity was measured 48 h after transfection. MiR-101 significantly suppressed firefly luciferase activity in cells transfected with the Wt *c-Met* 3′-UTR reporter construct ($^*P < 0.05$). (C) The *c-Met* expression level was significantly reduced following treatment of T24 cells with miR-101 mimics or siMet. ($^*P < 0.05$) (D) T24 cells were transfected with NC, miR-101 mimics or siMet. Western blot analysis was performed to detect expression of *c-Met* or GAPDH.

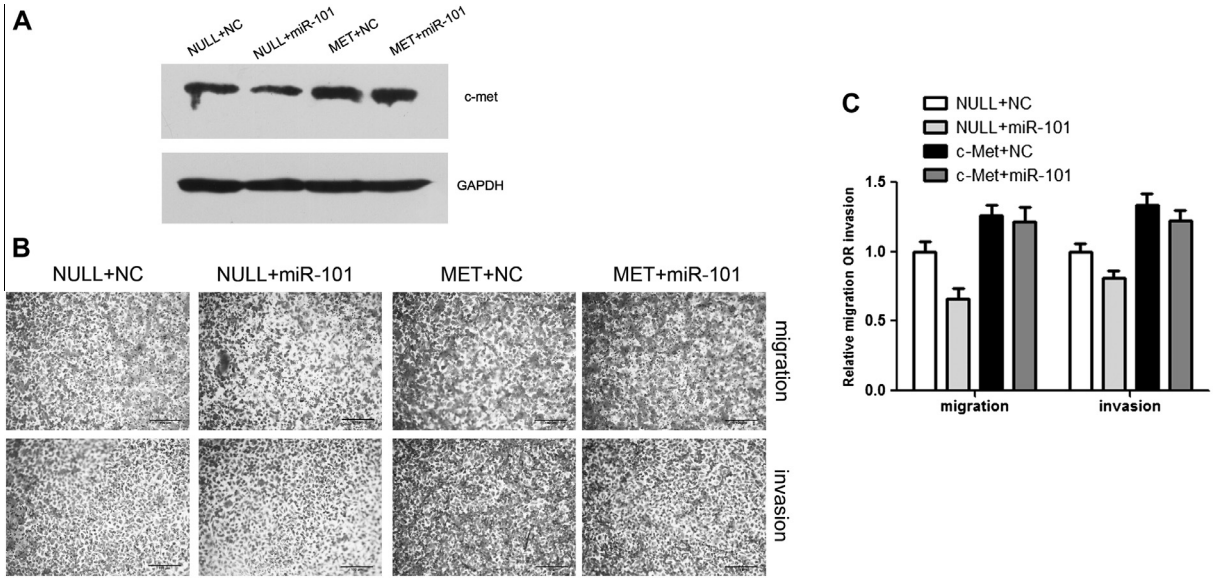

Fig. 4. Forced expression of *c-Met* rescues miR-101-dependent decreases in cell migration and invasion. (A) Either miR-101 mimics or NC oligos were co-transfected with pT-Met or the empty pTarget vector into T24 cells. Western blot analysis was then performed to detect expression of *c-Met* or GAPDH. (B) T24 cells were transfected as in (A) and then used in a transwell migration assay (top) or an invasion assay (bottom). (C) Representative migration and invasion results at 200× are shown in (B). Values are expressed as $\chi \pm s$ of 3 independent experiments performed in triplicate ($^*P < 0.05$).

reported up-regulation in bladder cancer [30]. As one of the various growth factors shown to be involved in bladder cancer progression, the HGF/*c-Met* pathway was demonstrated to play a key role in the epithelial–mesenchymal transition, invasiveness and metastasis of bladder cancer cells [31]. It has also been reported that *c-Met* is essential to the metastatic potential of bladder cancer cells and provides a powerful prognostic indicator for invasion and metastasis [4]. We therefore examined the effect of miR-101 on *c-Met* expression in bladder cancer cells.

In the present study, we demonstrated that miR-101 expression was significantly decreased in T24 human bladder cancer cells compared with normal bladder mucosa epithelial cells and in

human bladder cancer tissues versus normal adjacent tissues. We also found that forced miR-101 expression inhibited the capacities of T24 cells to migrate and invade, while forced *c-Met* expression partially reversed the inhibitory effects of miR-101 to promote migratory and invasive abilities. Importantly, we observed that forced miR-101 expression down-regulated *c-Met* mRNA and protein expression levels. Finally, we showed using a luciferase assay that miR-101 inhibited *c-Met* expression by directly targeting its 3′-UTR and that forced expression of *c-Met* partially rescued the effects of miR-101 on cell migration and invasion. To summarize, our study indicates that *c-Met* may be one of the direct targets of miR-101 involved in cell migration and invasion.

In conclusion, our study provides new evidence that miR-101 reduces bladder cancer cell migration and invasion, at least in part by regulating metastasis-promoting *c-Met*. Although further work is needed to identify other relevant targets of miR-101 in addition to *c-Met*, this study provides new insight into the function of miR-101 in bladder cancer and suggests that miR-101 could provide a novel molecular target for preventing invasion and metastasis of bladder cancers.

Acknowledgments

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