MicroRNA-10b promotes migration and invasion through KLF4 and HOXD10 in human bladder cancer

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Abstract. The present study was performed to investigate the effect of microRNA-10b (miR-10b) on cell migration and invasion in human bladder cancer (BC). Real-time PCR was performed to detect the expression of miR-10b in BC cell lines. miR-10b mimics, the negative control for mimics, miR-10b inhibitor and the negative control for inhibitor were transfected into BC cell lines and the effects of miR-10b on the migration and invasion of cells were investigated through Transwell assay. Meanwhile, protein levels of KLF4, HOXD10, E-cadherin and MMP14 were measured. Luciferase assays were also performed to validate KLF4 and HOXD10 as miR-10b targets. *In vivo* metastasis assay was performed to validate if miR-10b can promote BC cell line metastasis *in vivo*. miR-10b is significantly upregulated in BC cell lines and metastatic tissues. Increased miR-10b expression significantly enhanced BC cell migration and invasion, while decreased miR-10b expression reduced cell migration and invasion. *In vivo* metastasis assay demonstrated that overexpression of miR-10b markedly promoted BC metastasis. Moreover, KLF4 and HOXD10 were identified as direct targets of miR-10b in BC cells. Silencing of KLF4 or HOXD10 recapitulated the pro-metastatic function. Furthermore, we found that E-cadherin and MMP14 may be the downstream factors of KLF4 and HOXD10 in the suppression of BC metastasis by miR-10b. These data suggest that miR-10b may function as oncogenes in BC cells. Targeting these novel strategies, inhibition of miR-10b/KLF4/E-cadherin axis and miR-10b/HOXD10/MMP14 axis may be helpful as a therapeutic approach to block BC cell metastasis.

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Introduction

Bladder cancer (BC) is the most common urinary tract malignancy and the fifth most common malignancy in the developed countries. Each year, an estimated 105,000 and 71,000 new patients are diagnosed with BC in Europe and America, and ~28.5 and 20%, respectively, succumb to this disease (1). The major cause of mortality and relapse from BC is metastasis. Metastasis is a complex process comprised of multiple steps. In the metastatic cascade, local invasion can be considered an initial, essential step in the malignancy of carcinomas leading to the generation of a distant metastasis (2).

It has become evident that, in addition to alterations in protein-encoding genes, abnormalities in non-coding genes can also contribute to carcinogenesis (3,4). In particular, microRNAs (miRNAs), short single-stranded RNAs, have long been known to be important regulators of various cellular processes in gene expression at the post-transcriptional level (5,6). Deregulated miRNAs in cancer may function as either tumor suppressors or oncogenes and play a critical role in carcinogenesis (4). Furthermore, evidence indicates that miRNAs can be implicated in both the promotion and suppression of metastasis (7). For example, Ma *et al* found that miR-10b initiates invasion and metastasis in breast cancer (8). The present study provided the first evidence of miRNA in tumor metastasis. Subsequently, several additional miRNAs have been reported to act at various steps of metastasis. miR-21 stimulates cell invasion and metastasis in breast, colon cancer and gliomas (9,10); let-7 family members inhibit cell adhesion, migration and invasion in lung, gastric and breast cancer (11-13); and the miR-200 and miR-205 family inhibit cell migration and invasion by targeting ZEB1 and ZEB2, both of which are EMT-inducing transcription factors.

Notably, some miRNAs multitask by interacting with different target genes in various cellular contexts. miR-10b is a member of these miRNAs, which has been characterized as an oncogene in several human cancers by targeting critical cancer-related pathways. Reports have proven that miR-10b promotes breast cancer growth by Twist1-miR-10b-HOXD10 axis (8) and miR-10b can facilitate human glioma growth by targeting BCL2L11/Bim, TFAP2C/AP-2, CDKN1A/p21 and

CDKN2A/p16 (14). Recent studies indicate it can also enhance the migration and invasion of human esophageal cancer by targeting KLF4 (15). Our previous study also demonstrated that KLF4 was downregulated and may significantly inhibit cell migration and invasion through epithelial-mesenchymal transition (EMT) inhibition in BC cells (16).

The present study provides the first evidence of the role of miR-10b in BC metastasis and partially elucidates the molecular mechanism underlying the phenomenon. We identified that miR-10b had a high expression both in cell lines and metastatic tissues in BC. Furthermore, we verified the function of miR-10b in BC cell migration and invasion *in vitro* and *in vivo*. In addition, KLF4 and HOXD10 were identified as direct and functional targets of miR-10b in BC cells. Of note, ectopic expression of miR-10b induced the downregulation of invasion suppressor E-cadherin and improved the expression of MMP14, a member of the membrane-type matrix metalloproteinase (MT-MMP) subfamily involved in the breakdown of extracellular matrix in physiological or pathological processes. We further demonstrated that downregulation of the E-cadherin by miR-10b, through targeting KLF4, and upregulation of the MMP14, through targeting HOXD10, promoted migration and invasion in BC cells, which may contribute to the pro-metastatic role of miR-10b.

Materials and methods

Cell culture and tissue collection. BC cell lines T24, 5637, J82 and EJ were purchased from ATCC and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 maintained at 37˚C. SV-HUC-1 was also purchased from ATCC and maintained in DMEM/F-12 medium. SCaBER was purchased from AllCells, LLC (Shanghai, China) in MEM medium. Paired samples of primary BC, and lymph node metastatic tissues were obtained from patients who had undergone radical cystectomy surgery at Tongji Hospital, Wuhan, China. All samples were clinically and pathologically shown to be correctly labeled. The present study was approved by the Hospital's Protection of Human Subjects Committee, and informed consent was obtained from all patients.

RNA extraction and real-time RT-PCR. Total RNA, including miRNA, was extracted using the mirVana miRNA isolation kit (Ambion, Austin, TX, USA) following the manufacturer's protocol. The integrity of the RNA was examined with an RNA 6000 Nano Assay kit and a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The RT and PCR primers for miR-10b and U6 were purchased from RiboBio (Guangzhou, China). The PCR primers for KLF4 were: 5'-ACC CACACTTGTGATTACGC-3' and 5'-CCGTGTGTTTACGG TAGTGC-3'. The PCR primers for GAPDH were: 5'-ACCCA CACTTGTGATTACGC-3' and 5'-GTGTCGCTGTTGAAGT CAGA-3'. The first-strand cDNA was synthesized using the RT Reagent kit (Takara, Dalian, China). Real-time PCR was performed using SYBR Premix Ex Taq II (Takara) and measured in a LightCycler 480 System (Roche, Basel, Switzerland). Expression of U6 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control. All the reactions were run in triplicate.

Lentivirus infection and oligonucleotide transfection. The miR-10b lentivirus were purchased from GeneChem (Shanghai, China). The constructs containing the pre-miR-10b sequence, and 100 bases of upstream and downstream flanking these sequences were cloned into the pGCSIL-GFP vector. Target cells ($1x10^5$) were infected with $1x10^7$ lentivirus transducing units in the presence of 10 μg/ml polybrene. Empty lentiviral vector was used as negative control. The miR-10b inhibitor, mimic, negative control for mimic, negative control for inhibitor, negative control for siRNA and siRNA of KLF4, HOXD10 were designed and synthesized by RiboBio. Target cells were transfected with miR-10b inhibitor, mimic, siRNA or negative control using X-tremeGENE siRNA Transfection Reagent (Roche, Germany) according to the manufacturer's protocol.

Plasmid construction and dual-luciferase reporter assay. To construct a luciferase reporter vector, the wild-type 3'-UTR of KLF4 and HOXD10 were PCR-amplified using genomic DNA from 293T, GES and GC9811 cells as templates. The corresponding mutant constructs were created by mutating the seed regions of the miR-10b-binding sites. Both wild-type and mutant 3'-UTRs were cloned downstream of the luciferase gene in the psiCheck-2 luciferase vector. The constructs were verified by sequencing. For luciferase reporter assay, 5637 and EJ cells were seeded in 24-well plates and transiently transfected with appropriate reporter plasmid and miRNA using X-tremeGENEsiRNA Transfection Reagent. After 48 h, the cells were harvested and lysed, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Firefly-luciferase was used for normalization. For each plasmid construct, the transfection experiments were performed in triplicate.

Migration and invasion assay. For migration assay, infected or transfected cells were harvested and in serum-free RPMI-1640 medium, 5637 ~$1.5x10^5$ cells or EJ ~$5x10^4$ cells were placed into Boyden chambers (Corning, Cambridge, MA, USA) with an 8.0-mm pore membrane. For invasion assay, cells were placed into chambers coated with 150 mg of Matrigel (BD Biosciences, Bedford, MD, USA). The chambers were then inserted into the wells of a 24-well plate and incubated for 24 h in RPMI-1640 medium with 10% FBS before examination. The cells remaining on the upper surface of the membranes were removed, whereas the cells adhering to the lower surface were fixed, stained in a dye solution containing 0.5% crystal violet and counted under a microscope (Olympus Corp., Tokyo, Japan) to calculate their relative numbers. The results were averaged among three independent experiments.

Western blotting. Whole-cell lysates were prepared in NP-40 buffer (Byotime, Haimen, China), and western blotting was performed as previously described. The primary antibodies used were KLF4 (Abcam, Hong Kong, China), E-cadherin, HOXD10 (both from Cell Signaling Technology), MMP14 and GAPDH (both from Boster, Wuhan, China).

In vivo metastasis assay. For *in vivo* metastasis assay, $1x10^6$ EJ cells infected with miR-10b lentivirus and negative control containing GFP label were suspended in 200 μl phosphate-buffered saline and injected into the tail vein of nude mice

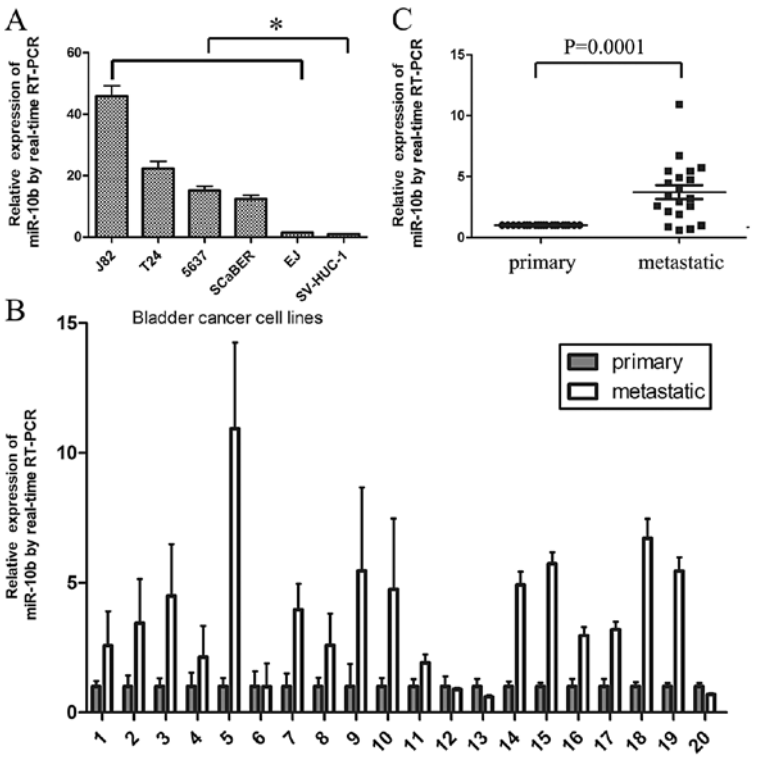

Figure 1. Expression of miR-10b in BC cell lines and tissue samples. (A) miR-10b expression in six bladder cell lines was measured by real-time quantitative RT-PCR, and the U6 small nuclear RNA was used as an internal control (means ± SEM; n=3). (B) The relative expression of miR-10b in primary BC tissues and lymph node metastatic tissues from 20 patients (means ± SEM; n=3). (C) The lymph node metastatic tissues had a higher expression of miR-10b than primary BC tissues with a P<0.01 (means ± SEM; n=3). BC, bladder cancer.

(three in each group). Mice were sacrificed and lungs were resected 4 weeks after injection. The incidence and volume of metastases were estimated by the imaging of mice for bioluminescence using the LivingImage software (Xenogen, Baltimore, MD, USA). The photon emission level was used to assess the relative tumor burden in the mouse lungs. All animal studies were conducted under approved guidelines of the Animal Care and Use Committee of Tongji Hospital (Wuhan, China).

Statistical analysis. The SPSS 18.0 program (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Experimental data are expressed as the means ± SE. The differences between groups were analyzed using the Student's t-test when comparing only two groups or they were assessed by one-way analysis of variance when more than two groups were compared. For comparison of paired tissues, a paired Student's t-test was used to determine the statistical significance. Differences were considered statistically significant at $P<0.05$, $^{*}P<0.05$.

Results

miR-10b is upregulated in BC cell lines and human BC metastatic tissue samples. miR-10b expression was detected in five human BC cell lines T24, 5637, J82, EJ, SCaBER and SV-HUC-1, an immortalized bladder epithelial cell line. Quantitative reverse-transcription PCR (qRT-PCR) showed that miR-10b expression was significantly increased in BC cell lines compared with SV-HUC-1 (Fig. 1A). Ma *et al* (8) found that miR-10b was highly expressed in metastatic breast cancer cells and positively regulated cell migration and invasion. In the present study, miR-10b was also detected in 20 paired primary tumors and their related metastatic lymph nodes. Result indicated that miR-10b was expressed higher in metastatic lymph nodes than in primary tumors in 16/20 matched specimens with a P-value of 0.0001 (Fig. 1B and C).

miR-10b suppresses BC cell migration and invasion in vitro. Transwell assay was performed to investigate whether miR-10b enhance BC cell migration and invasion. First, miR-10b inhibitor was transfected into 5637 and EJ cells to reduce the endogenous miR-10b expression. Expression of miR-10b was suppressed in the transfectant. The results showed that the inhibition of miR-10b expression led to a 40.7 and 44.2% reduction in migration and invasion compared to the control in 5637 cells; a similar 45.0 and 48.6% reduction in migration and invasion was also found in another BC cancer cell line EJ (Fig. 2). Second, miR-10b mimics were transfected to upregulate the level of miR-10b in 5637 and EJ cells, which led to a marked increase in migration and invasion (Fig. 2). Taken together, these results indicate that miR-10b promotes BC cell migration and invasion *in vitro*.

KLF4 and HOXD10 mRNA are direct targets of miR-10b. KLF4 and HOXD10, which have putative binding sites for miR-10b respectively, have been reported to be the direct targets of miR-10b in other types of cancer. However, these target effects were not observed in BC. PsiCheck-2-KLF4 3'-UTR, PsiCheck-2-KLF4-3'-UTR-mut, PsiCheck-2-HOXD10-3'-UTR and PsiCheck-2-HOXD10-3'-UTR-mut were

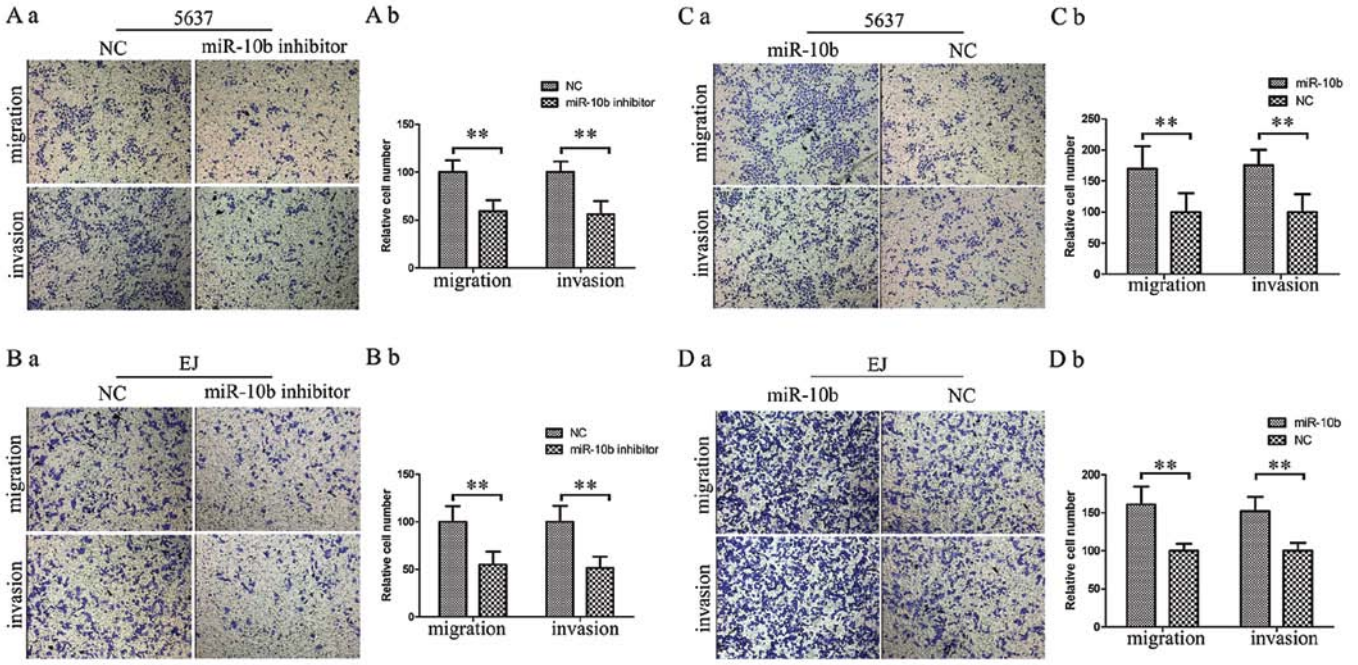

Figure 2. miR-10b promotes migration and invasion of human BC cell lines *in vitro*. (A and B) Transwell migration and invasion assays using 5637 and EJ cells transfected with miR-10b inhibitors or negative control (NC). Representative images are shown on the left, and the quantification of 10 randomly selected fields is shown on the right (means ± SEM; n=3; **P<0.01). (C and D) Transwell migration and invasion assays using 5637 and EJ cells transfected with miR-10b mimics or NC. Representative images are shown on the left, and the quantification of 10 randomly selected fields is shown on the right (means ± SEM; n=3; **p<0.01). BC, bladder cancer.

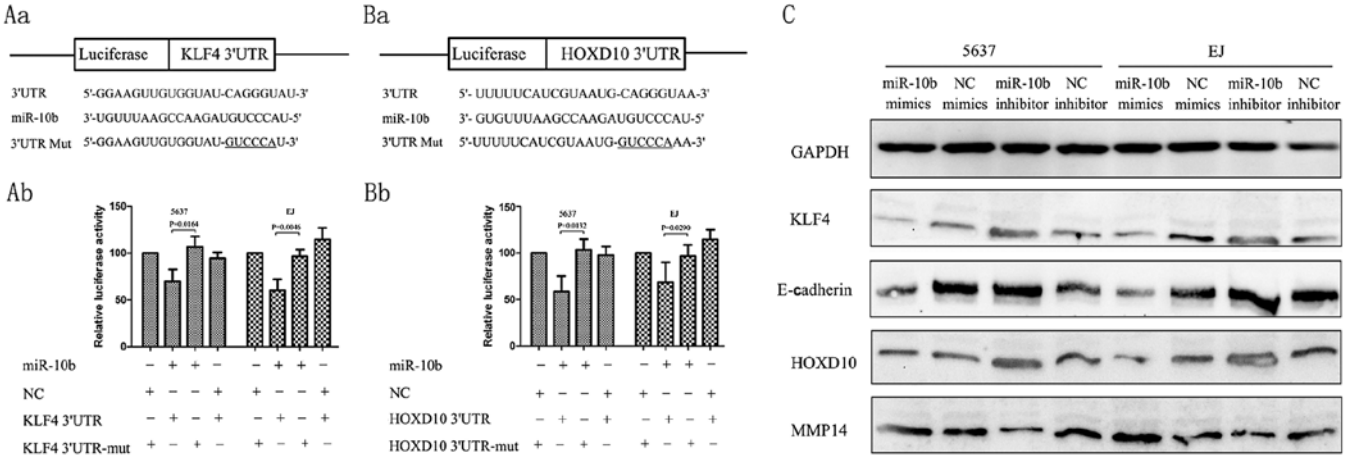

Figure 3. KLF4 and HOXD10 mRNA are direct targets of miR-10b. (Aa and Ba) Diagram of the luciferase reporter plasmids: plasmid with the KLF4-3'-UTR insert, HOXD10-3'-UTR insert and plasmid with a mutant KLF4-3'-UTR-mut or HOXD10-3'-UTR-mut which carried a substitution of six nucleotides within the miR-10b binding site. (Ab and Bb) Luciferase activity assay demonstrated a direct targeting of the 3'-UTR of KLF4 and HOXD10 by miR-10b. Cells (5637 and EJ) were transfected with miR-10b mimics and PsiCheck2-KLF4-3'-UTR/PsiCheck2-KLF4-3'-UTR-mut or PsiCheck2-HOXD10-3'-UTR/PsiCheck2-HOXD10-3'-UTR-mut. Firefly was used for normalization of transfection efficiency. After 48 h, the luciferase activities were measured (means ± SEM; n=3). (C) Cells (5637 and EJ) were transfected with miR-10b mimics, the negative control (NC) for mimics, miR-10b inhibitor or the NC for inhibitor and western blotting was performed 48 h after transfection.

constructed (Fig. 3Aa and Ba) to examine whether KLF4 and HOXD10 mRNA are direct targets of miR-10b in renal cancer cells. Co-transfection of 5637 cells with PsiCheck-2-KLF4 3'-UTR and miR-10b mimics led to a 30.0% decrease in the luciferase activity compared with the negative control. Co-transfection of 5637 with PsiCheck-2-HOXD10-3'-UTR and miR-10b mimics also led to a 42% decrease in the luciferase activity. This suppression was rescued by the six-nucleotide substitution in the core binding sites. A similar effect was also found in EJ cells (Fig. 3). Real-time qPCR of KLF4 mRNA showed that miR-10b overexpression or inhibition had little effect on KLF4 or HOXD10 mRNA level (data not shown). Collectively, these results suggested that miR-10b regulates KLF4 and HOXD10 expression in renal cancer cells by directly targeting those 3'-UTR through post-transcriptional regulation way.

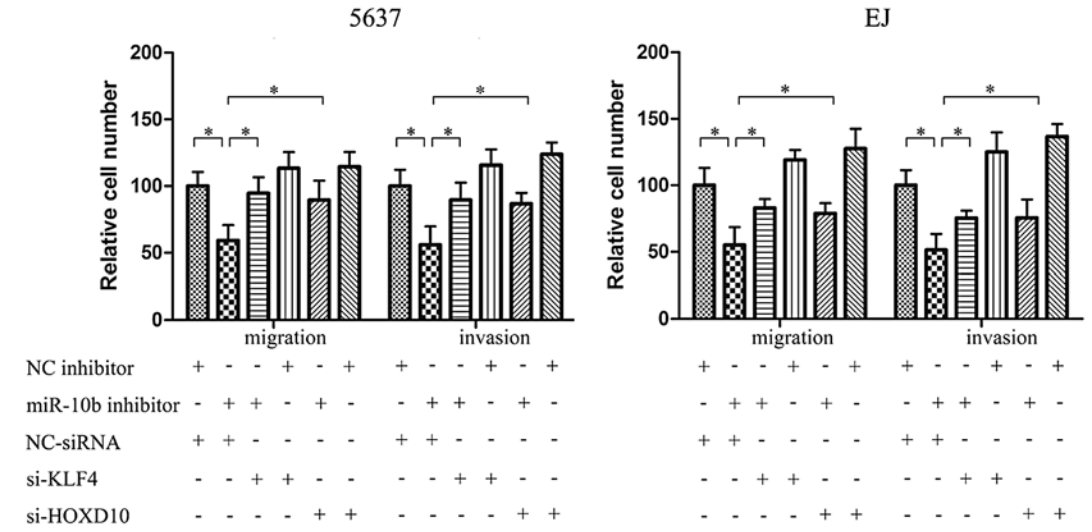

Figure 4. KLF4 and HOXD10 partly suppress migration and invasion initiated by miR-10b. KLF4 and HOXD10 inhibit BC cell migration and invasion *in vitro*. Transwell assay of 5637 and EJ cell migration and invasion after co-transfection (means ± SEM; n=3). NC inhibitor, negative control for inhibitor. NC-siRNA, negative control for siRNA. BC, bladder cancer.

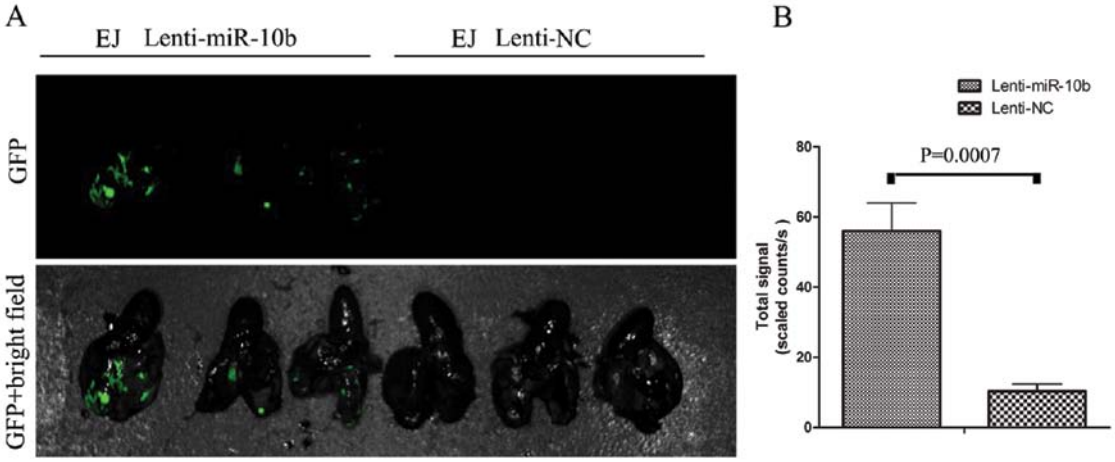

Figure 5. miR-10b promotes BC cell metastasis *in vivo*. (A) Representative bioluminescent images of lungs of nude mice at the 4th week after I.V. injection of BC EJ cells. (B) Quantification analysis of fluorescence signal from captured bioluminescence images. BC, bladder cancer.

Immunoblot assay showed that the overexpression of miR-10b decreased the endogenous expression of protein of KLF4 and HOXD10, whereas downregulation of miR-10b with an anti-miR-10b inhibitor increased the expression of KLF4 and HOXD10. E-cadherin is a central component involved in the conversion between mesenchymal and epithelial phenotypes, and KLF4 controls the expression of E-cadherin by reducing the expression of snail (17), slug (18,19) or directly binding to the GC-rich/E-box region of the E-cadherin promoter (20). In the present study, ectopic overexpression of miR-10b reduced the expression of E-cadherin in BC cell lines (Fig. 3C). Tumor invasive factors MMP14 have been reported to be directly regulated by HOXD10 (21). Then, we examined the levels of MMP14 after transfection with miR-10b inhibitors and the results showed that the protein was downregulated by the miR-10b inhibitors, whereas transfection with the miR-10b mimics may upregulate MMP14 expression levels (Fig. 3C).

Silencing KLF4 and HOXD10 expression efficiently blocks the effect of miR-10b downregulation on BC cell migration and invasion. We then tested whether miR-10b inhibits BC cell migration and invasion through targeting KLF4 and HOXD10. Small interfering RNA target KLF4 and HOXD10 were used to knock down endogenous KLF4 and HOXD10. Using Transwell assay, the inhibition effect of migration and invasion by miR-10b inhibitor in 5637 cells may be partially prevented by KLF4 or HOXD10 knockdown with siRNA-KLF4 or siRNA-HOXD10 to varying degrees (Fig. 4). The same effect was also identified in EJ cells.

miR-10b suppresses BC cell metastasis in vivo. To further determine whether miR-10b promotes metastatic behaviors *in vivo*, EJ cells stably transfected by Lenti-miR-10b were delivered into nude mice through tail vein injection. Bioluminescence imaging taken 28 days later revealed that the fluorescence signal in the Lenti-miR-10b group was significantly stronger

than in the Lenti-NC group, indicating that more metastasis formed in the lung after miR-10b overexpression (Fig. 5). This assay *in vivo* suggested that miR-10b has a potential to promote metastasis in BC cells.

Discussion

MicroRNAs (miRNAs) have emerged as important regulators of gene expression at the post-transcriptional level and regulate a wide range of physiological and developmental processes. Over the past few years, it has become clear that alterations in the expression of miRNAs contribute to the pathogenesis of most human cancers, where they act as either oncogenes or tumor suppressors (22).

Aberrant miR-10b expression is correlated with carcinogenesis. miR-10b plays an oncogenic effect in breast cancer, neurofibromatosis type 1, oral, colorectal, gastric, pancreatic, human glioma cancer, human nasopharyngeal carcinoma and human esophageal cancer (14,15,23-29). Ma *et al* first discovered that miR-10b initiates breast cancer invasion and metastasis (8). Nishida *et al* showed that transfection with miR-10b confers resistance to the chemotherapeutic agent 5-fluorouracil in colorectal cancer cells (28). miR-10b also reduces glioma cell growth by cell cycle arrest and apoptosis (14). In the present study, we investigated the role and the functional targets of miR-10b in human bladder cancer (BC). miR-10b expression was detected in several BC cell lines, an immortal urothelial cell line SV-HUC-1 and bladder tissue by RT-PCR. miR-10b expression was significantly increased in all five BC cell lines. Furthermore, miR-10b expression in primary bladder tumor tissue was lower than that in the metastatic tissues. Then, upregulated miR-10b expression may significantly promote BC cell migration and invasion *in vitro* and *in vivo*. However, there was no obvious evidence that miR-10b promotes BC cell proliferation or confers resistance to the chemotherapeutic agent 5-fluorouracil or cisplatin (data not shown), which had been reported in other types of cancer.

KLF4 and HOXD10, which have putative binding sites for miR-10b have been reported to be direct targets of miR-10b in other types of cancer. However, these target effects were not observed in BC.

KLF4 is a zinc-finger transcription factor that regulates a multitude of processes in normal tissue including proliferation, differentiation, apoptosis, tissue homeostasis and self-renewal (30-34). Inactivation or silencing of KLF4 has been observed in a number of human cancers including colorectal, gastric, pancreas, esophageal, lung, prostate and hepatocellular cancer (35-42), which suggests KLF4 may function as a tumor suppressor and a potential prognostic marker for overall survival time and lymph node metastasis (35,40). The present study reported that KLF4 was downregulated partially by promoter methylation and has a function as a tumor suppressor gene in renal clear cell cancer and bladder urothelial cancer. However, KLF4 in BC has never been reported to be connected with miR-10b. In the present study, we used luciferase reporter assay to identify that KLF4 was also the direct target of miR-10b in BC cells; we not only found the mediate role in the promotion of migration and invasion of miR-10b, but we also showed another cause of KLF4 downregulation besides promoter methylation (16). HOXD10, a member of type I class homeobox (Hox) genes, plays an important role in suppressing angiogenesis and maintaining a quiescent, differentiated phenotype in endothelial cells (21). Specifically, HOXD10 suppresses expression of genes that directly affect remodeling of the extracellular matrix and cell migration during angiogenesis such as a3 integrin, matrix metalloproteinase 14 (MMP14), and urokinase-type plasminogen activator receptor (uPAR). To date, there is no report on the function of HOXD10 in BC. In the present study, we found that the siRNA for HOXD10 can promote the migration and invasion of BC cells. Taken together, these results established a functional connection between miR-10b, KLF4 and HOXD10, and confirmed that miR-10b functions as a pro-metastatic miRNA in BC cells by targeting KLF4 and HOXD10.

Metastasis arises through a multistep process that begins when cancer cells within tumors *in situ* detach from neighboring cells and invade the basement membrane (43). The best characterized alteration mainly involved the loss of E-cadherin, a key cell-to-cell adhesion molecule (44). Expression of E-cadherin is controlled by several transcriptional repressors, including Twist, Snail1, Snail2/Slug, E47, ZEB1/TCF8 and ZEB2/SIP1, which bind to E-boxes in the E-cadherin promoter. Yori *et al* (20) found that KLF4 silencing increased Snail expression in breast cancer. However, Liu *et al* (19) proved that KLF4 reverses epithelial-mesenchymal transition (EMT) through downregulation of Slug not snail in prostate cancer. Yori *et al* also found that E-cadherin is a novel transcriptional target of KLF4. Our previous study also reported that KLF4 overexpression inhibits urothelial cancer cell EMT, a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal cells, and promote the expression of cell-cell adhesion markers such as E-cadherin (16). In the present study, miR-10b inhibitor transfection in BC was sufficient to promote E-cadherin expression. Ibrahim *et al* found that miR-10b can downregulate E-cadherin by miR-10b-syndecan-1 axis (45). Our report suggested that promotion of E-cadherin by miR-10b, through targeting KLF4, can promote BC cell metastasis in the early stage.

In summary, miR-10b was significantly upregulated in highly metastatic cells and tissues. miR-10b overexpression can enhance the migration, invasion and metastasis ability of BC cells *in vitro* and *in vivo*. Additionally, KLF4 and HOXD10 were found to be direct and functional targets of miR-10b, and mediated the effect in BC cell migration and invasion.

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