

Original Article

Epigenetically induced silence of SOX10 promotes tumor proliferation and invasion in bladder cancer

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Abstract: Epigenetic alteration induced loss function of the SOX10 has been associated with different types of human cancers. However, the epigenetic regulation and molecular function of SOX10 in bladder cancer remain unknown. In this study we investigate the role of SOX10 in the progression of bladder cancer. SOX10 expression was analyzed in 50 paired bladder cancer and adjacent non-tumor tissues. Decreased SOX10 was found in 38% (19/50) of bladder cancer and was correlated with lymph node metastasis. Pharmacologic demethylation reactivated SOX10 expression along with concomitant promoter demethylation in bladder cancer cell lines. Cellular function experiments revealed that restoration of SOX10 inhibited cell proliferation, invasion and migration. Our results suggest that SOX10 may function as a tumor suppressor gene and provide new insight into the molecular mechanisms leading to bladder cancer.

Keywords: SOX10, bladder cancer, methylation, invasion

Introduction

Bladder cancer is one of the most common cancers and causes approximately 150,000 deaths annually world-wide. Despite of the improvements made over the years to its treatment, the prognosis for bladder cancer remains poor because of metastasis and the development of resistance to chemotherapy [1]. A better understanding the mechanisms leads to bladder cancer progression will be fundamental to develop novel therapeutic strategies.

Sex-determining region Y-box 10 (SOX10) is a member of the SOX family of transcription factors which are characterized by the evolutionarily conserved high mobility group (HMG) box domains [2]. The HMG box mediates the DNA-binding properties of SOX proteins with different levels of efficiency. SOX10 plays an important role in diverse biologic processes including normal embryogenesis, neural crest development and differentiation, as well as in the development of specific cell types and lineages within the central nervous system [3, 4].

Abnormalities (over- or under- expression, or genetic mutations) of SOX10 have been shown to play critical roles in cancer formation and development. SOX10 may act as both an oncogene and a tumor suppressor depending on tumor origin. SOX10 was reported to possess tumor-promoting activities in melanoma [5] and gliomas [6]. On the other hand, decreased expression of SOX10 was found to promote tumor cell progression of Merlin-null schwannoma cells [7]. It has been demonstrated that methylation induced silence of SOX10 in multiple digestive cancer including colorectal cancer, gastric cancer and esophageal carcinoma. SOX10 suppressed β -catenin signaling, as well as the epithelial-mesenchymal transition (EMT) and stemness of tumor cells, further leading to the inhibition of tumor cell migration/invasion [8]. However, the role of SOX10 in bladder cancer remains unknown. In this study, we analyzed the expression of SOX10 in human bladder cancer cell lines and bladder cancer tissues. We also characterized the tumor-suppressive effects of SOX10 in bladder cancer.

Materials and methods

Cell lines and plasmids

The human bladder cancer cell line, T24 and 5637, were cultured at 37°C in an atmosphere containing 5% CO₂ in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. V5-tagged SOX10 was constructed by cloning full-length cDNA into pLVX-AcGFP-N1 vectors (Clontech, Mountain View, CA), designated as LV-SOX10, and with empty vector as negative control (designated as LV-GFP) as described previously [9].

Clinical samples

A total of 50 paired bladder cancer tissues and adjacent non-tumor tissue were obtained from PLA General Hospital. The use of all the human samples and the experimental procedures for this study were reviewed and approved by the hospital ethics committees. Prior written informed consent was also obtained from each bladder cancer patient in this study. All these bladder cancer patients were not received other therapeutic treatments before surgery. All tissues were immediately snap-frozen at liquid nitrogen and transferred to -80°C refrigerator.

Immunohistochemistry (IHC)

The expressions of SOX10 protein in the specimens were detected by Dako REAL™ EnVision™ Detection System (DAKO) as described previously [10]. Briefly, sections were cut at 4 μm and dried at 60°C. The sections were deparaffinized in xylene, hydrated through graded alcohols and rinsed in distilled water. Heat-induced epitope retrieval was performed in a pressure cooker in 10 mM citrate buffer (pH 6.0) for 5 min. Sections were cooled for 30 min and then were rinsed in PBS. Endogenous peroxidase activity was blocked by hydrogen peroxide treatment. The antibody against SOX10 was diluted at 1:100 and incubated overnight at 4°C. Secondary detection was accomplished using horseradish peroxidase-labelled secondary anti-mouse antibody and staining was visualized using the peroxide substrate solution diaminobenzidine followed by light nuclear counterstaining with hematoxylin. Two observers evaluated the staining pattern of the protein separately and scored each specimen

according to the percent of positive cells identified. An average of 20 fields was observed per specimen. Tissue sample immunoreactivity was considered negative (-) when fewer than 10% of cells showed the same staining intensity as the positive control in arterial endothelium, low (+) when 11% to 20% showed such staining, medium (++) when 21% to 60% cells showed such staining and high (+++) when more than 60% showed such staining.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA from tissues or cells was isolated with Trizol reagent (Invitrogen, CA). First-strand cDNA was generated using the PrimeScript RT reagent Kit (TAKARA). Analysis of mRNA levels was performed on a 7500 Real-Time PCR System (Applied Biosystems) with SYBR Green-based real-time PCR. GAPDH was used as an endogenous control to normalize the amount of total RNA in each sample. Relative expression of genes was calculated and expressed as 2^{-ΔΔCT}. The primer sequences of SOX10 in this study were SOX10F: 5'-CACTACAAGAGCGCCCACT-3', SOX10R: 5'-TGCCGAAGTCGATGTGAGG-3'; GAPDH-F: 5'-GATGACCTGCCACAGCCT-3', GAPDH-R: 5'-ATCTCTGCCCCCTCTGCTGA-3'.

Western blot

Total cell lysate was prepared in 1 × SDS lysis buffer (62.5 mM Tris-HCl, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue). Proteins at the same amount were separated by 10% SDS-PAGE and transferred onto PVDF membranes. After probing with antibodies, the signals were visualized by Immobilon™ Western Chemiluminescence HRP Substrate (Millipore, Billerica, MA). The antibodies used in this study were: GAPDH-HRP (Kangchen Biotechnology), Anti-mouse IgG-HRP (P0161), Monoclonal SOX10 antibody (clone 20B7, MAB 2864, R&D Systems).

5-Aza-2'-deoxycytidine treatment

Dissolve 10 mg of 5-Aza-2-Deoxycytidine (5-Aza-2'dC) (Sigma-Aldrich) in H₂O. The treatment was performed using 10 μM of 5-Aza-2'dC for 3 days with change of Aza-containing medium every 24 hours. Cells were then harvested for RNA extractions.

Decreased SOX10 in bladder cancer

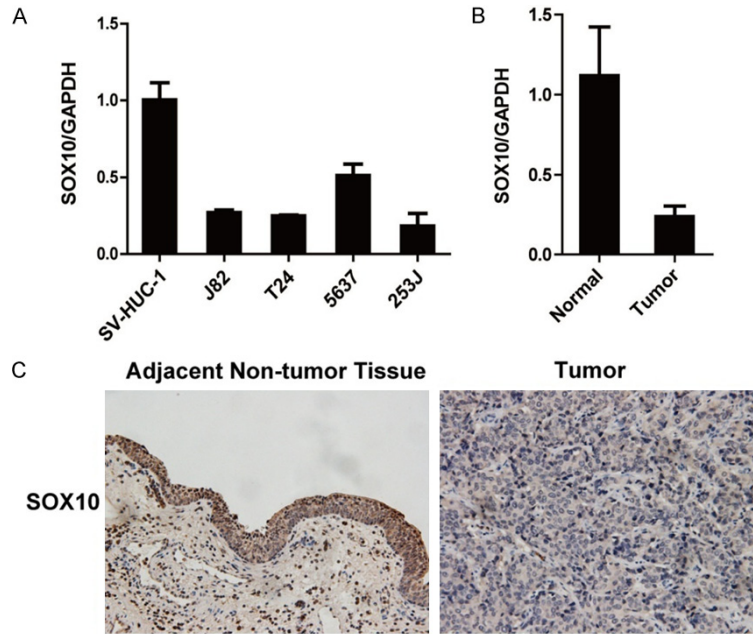


Figure 1. A. Expression of SOX10 in bladder cancer cells was analyzed by real-time PCR (qRT-PCR). B. Representative qRT-PCR results in normal and tumor tissues. C. Immunohistochemistry analysis of SOX10 expression in bladder cancer and adjacent non-tumor tissue (original magnification $\times 200$).

Table 1. Association between SOX10 expression and clinicopathological variables

Clinicopathological variables	Bladder cancer (n = 50)		P-value
	Low expression	High expression	
Age (years)			
<60	11	16	0.6653
≥ 60	8	15	
Gender			
Male	13	17	0.3413
Female	6	14	
pT stage			
pTa	11	10	0.0746
pT1-pT2	8	21	
pN stage			
-	6	21	0.0128
+	13	10	
Histological Grade			
G1	7	15	0.4247
G2-3	12	16	
Tumor multiplicity			
Yes	10	11	0.2331
No	9	20	

MTS assay

Cell growth was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-6915

(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega, Madison, WI) in 96-well plates (1000 cells per well) following the instructions of the manufacturer. Each experiment was done in triplicate and repeated three times.

Colony formation assay

Cells infected with LV-GFP or LV-SOX10 were counted and cultured in a 6-well plate (1.0×10^3 per well) for 10 to 15 days. Surviving colonies were stained with gentian violet after methanol fixation and visible colonies were counted.

Transwell migration and invasion assays

Transwell migration assays were quantified in vitro using

Transwell chambers with polycarbonate membrane filters (8 μm pore size; Corning, NY) according to the manufacturer's instructions. In brief, the lower chamber was filled with 0.6 ml medium containing 20% fetal bovine serum, and 0.2 ml of medium that contained 3×10^5 cells under serum-starving conditions was plated in the upper chamber and incubated at 37°C for 48 hours. Then cells that had not migrated were removed from the upper face of filters using cotton swabs. The cells that migrated through the membrane and attached to the bottom of the membrane were fixed and stained with crystal violet. Images of five random fields were captured from each membrane and the number of migratory cells was counted, and the extent of migration was expressed as the average number of cells per microscopic field at a magnification of 100. The mean of triplicate assays for each experimental condition was used. Similar inserts coated with Matrigel (Chemicon International, Temecula, CA) were used to determine invasive potential in invasion assay. Two independent investigators were blinded when reading the migration and invasion assays.

Wound-healing assay

For wound-healing assay, the cells were first seeded in 6-well culture plates. A wound was

Decreased SOX10 in bladder cancer

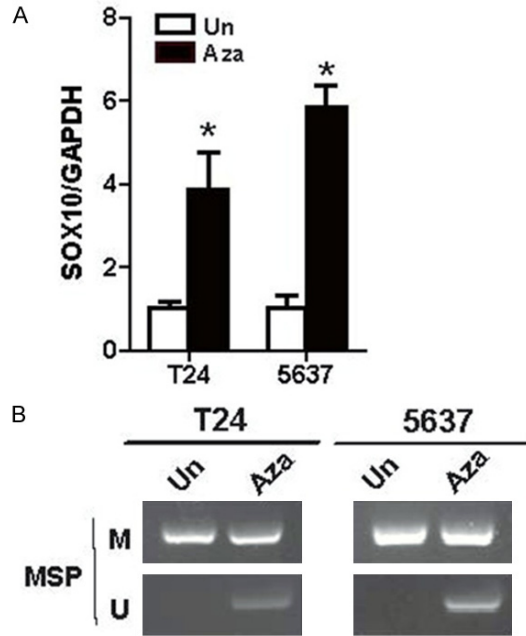


Figure 2. A. Expression of SOX10 mRNA in T24 and 5637 cells treated with 5-Aza related to untreated cells was determined by qRT-PCR. $**P < 0.01$. B. The methylation status of SOX10 in T24 and 5637 cells treated with 5-Aza was determined by methylation-specific PCR (MSP). Un, Untreated; M, Methylated; U, Unmethylated. $*P < 0.05$.

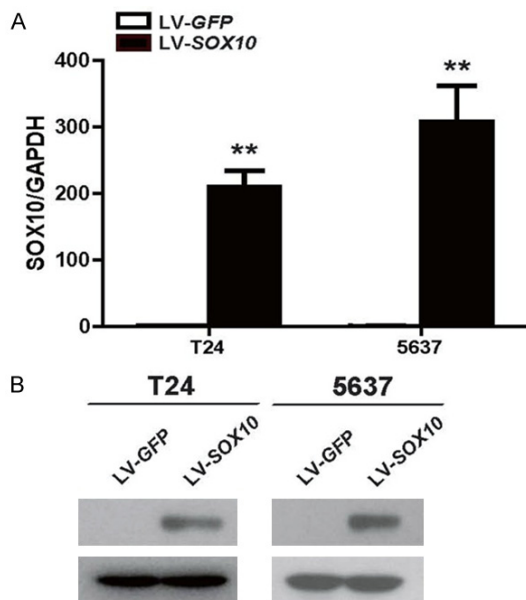


Figure 3. T24 and 5637 cells were infected with LV-SOX10 and LV-GFP as control. Expressions of SOX10 were detected by qRT-PCR. $**P < 0.01$ (A) and western blot (B).

made in the confluent monolayer with a plastic pipette tip and the migration of the cells at the

wound front was photographed using an inverted microscope at indicated times after the scratch.

Animal experiments

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of PLA General Hospital. T24 cells were infected with LV-GFP and LV-SOX10 and 5×10^6 cells were injected subcutaneously into each mouse ($n = 6$ mice/group). Tumor growth was monitored every 5 days by measuring tumor diameters. Tumor width (W) and length (L) were measured, and tumor volume was calculated using the following formula: $V = \text{length} \times \text{width}^2 \times 0.5$.

Statistical analysis

The analyses were carried out using SPSS 16.0 for Windows software. Pearson chi-square tests were used to evaluate the relationship between the expression of SOX10 and clinicopathological variables. Student's t test was used to determine the differences between groups. Data were presented as the mean \pm SEM. All statistical tests were two-sided, and $P < 0.05$ was considered statistically significant.

Results

Decreased expression of SOX10 in bladder cancer

First, we detected the levels of SOX10 mRNA and protein in 4 bladder cancer cell lines as well as urothelial immortal cell line SV-HUC-1 (**Figure 1A**). The level of SOX10 mRNA was relatively high in SV-HUC-1 and significantly decreased in 4 bladder cancer cell lines. Next, the expression of SOX10 was analyzed in a limited set of tissue samples (10 normal and 10 tumor tissues) by qRT-PCR. The expression of SOX10 was downregulated in bladder cancer tissue compared with non-tumor tissue (**Figure 1B**). Finally, we performed immunohistochemistry (IHC) to examine SOX10 protein expression in bladder cancer and paired adjacent non-tumor tissues using a monoclonal antibody described previously. In the non-tumor tissues, immunopositive staining of SOX10 was detected in the nuclear of urothelial epithelial cells (**Figure 1C**), whereas it was weakly detected in tumor tissues from the same patients. Weak immunostaining pictures of SOX10 were found in 19 of 50 (38%) cases of bladder cancer. The

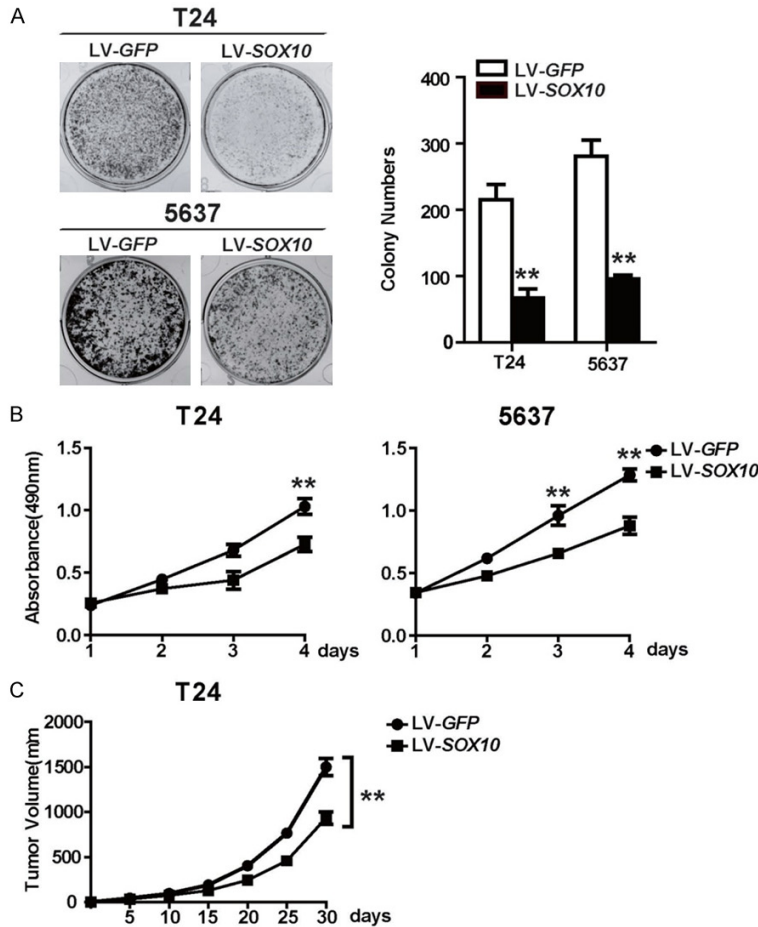


Figure 4. Ectopic expression of SOX10 suppresses bladder cancer proliferation. A. Representative of colony formation assays. Experiments were performed in triplicate three times. $**P < 0.01$. B. MTS cell proliferation assay for LV-GFP and LV-SOX10 infected T24 and 5637 cells. Values are mean \pm SEM for triplicate samples from a representative experiment. C. T24 cells infected with LV-SOX10 or LV-GFP were injected subcutaneously into nude mice. Tumor volume of each group was scored every 5 days.

correlations of SOX10 expression and clinicopathologic features were further analyzed. There was no significant correlation between SOX10 expression with age, gender, histological grade or tumor multiplicity. However, SOX10 expression was inversely associated with lymph node metastasis ($P = 0.0195$, **Table 1**), suggesting that low expression of SOX10 may be associated with the progression of bladder cancer.

Restoration of SOX10 expression by demethylation

It has been reported that SOX10 promoter contains a typical CpG island and promoter methylation mediates transcriptional silencing of

SOX10 in digestive cancers [9]. To test if decreased expression of SOX10 in bladder cancer is due to promoter methylation, T24 and 5637 were treated with DNA demethylating agent 5-Aza-2'dC, and then methylation specific PCR (MSP) and QRT-PCR were performed. The results showed that treatment with 5-Aza-2'dC increased SOX10 mRNA expression (**Figure 2A**) and decreased the methylated MSP products in both cell lines (**Figure 2B**). These data demonstrate that hypermethylation of CpG islands results in epigenetic silencing of SOX10 in bladder cancer cell lines.

SOX10 suppresses proliferation of bladder cancer cell lines

Previous data suggest that decreased expression of SOX10 may play an important role in the development of bladder cancer. To evaluate the function of SOX10 in bladder cancer cells, we employed lentivirus to elevate SOX10 expression (LV-SOX10) in T24 and 5637 cells. As shown in **Figure 3A** and **3B**, the expression of SOX10 was significantly elevated in T24 and 5637

cells at the mRNA and protein level, which was measured by qRT-PCR and western blot.

First, we compared the growth characteristics of T24 and 5637 cell lines with silenced SOX10 before and after ectopic expression of SOX10 by monolayer colony formation assay. The number of colonies formed by SOX10 re-expressed cells was significantly less than that with empty vector (**Figure 4A**). Besides, MTS assay revealed that LV-SOX10 significantly suppressed cell growth as compared with LV-GFP treatment (**Figure 4B**). To further confirmed the inhibitory effects of SOX10 on tumor growth *in vivo*, T24 cells infected with LV-SOX10 or LV-GFP were implanted subcutaneously into the flank of nude mice. As shown in **Figure 4C**,

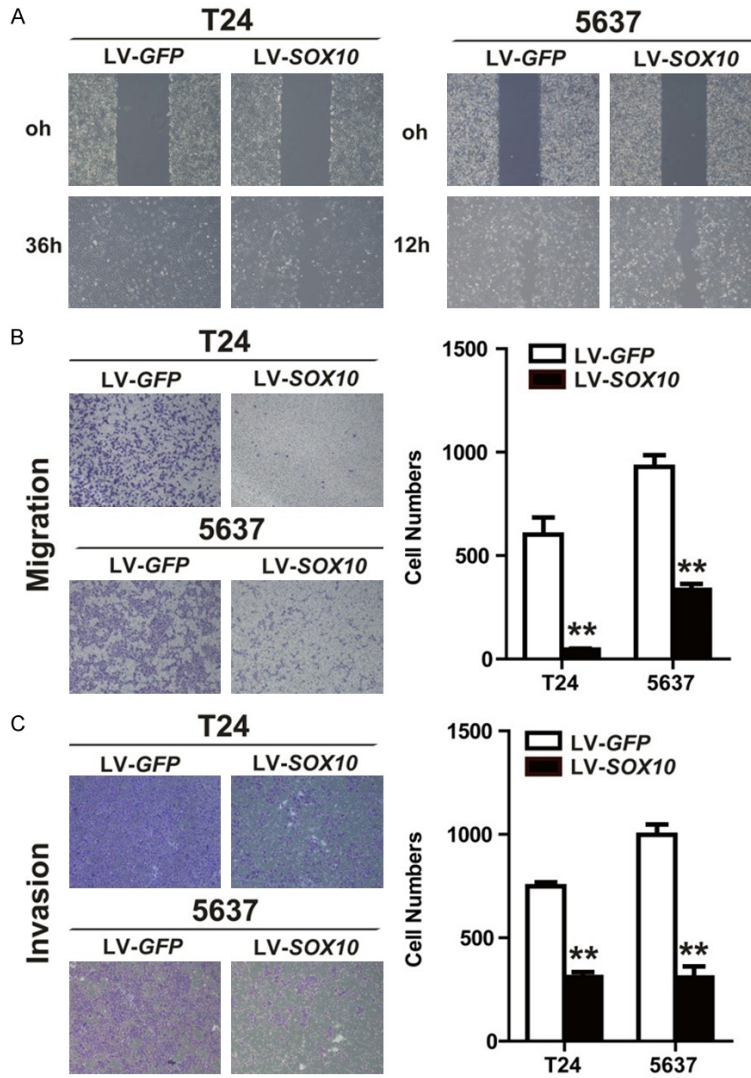


Figure 5. Ectopic expression of SOX10 suppresses bladder cancer migration and invasion. (A) Motilities of T24 and 5637 cells infected with LV-SOX10 or LV-GFP were examined by an *in vitro* wound healing assay. T24 and 5637 cells infected with LV-SOX10 or LV-GFP were incubated with or without matrigel for 24 or 48 hours (B and C). Cell numbers were determined by the average count of five random microscopic fields. Values shown are means \pm SEM (right) $**P < 0.01$.

5A). Consistently, Boyden chamber assays showed ectopic expression of SOX10 dramatically suppressed the migratory ability of bladder cancer cells (Figure 5B). Finally, we employed Boyden chamber assays by plating cancer cells on inserts coated with Matrigel to test the invasive potential of bladder cancer cells infected with LV-SOX10, and found that enforcing SOX10 expression in bladder cancer cells inhibited their invasion (Figure 5C). Altogether, these data suggest that SOX10 suppresses the motility and invasiveness of bladder cancer cells.

Discussion

In this study, we found that while SOX10 is highly expressed in bladder urothelial epithelium, it is frequently silenced or downregulated in bladder cancer cell lines and primary tumors. Decreased expression of SOX10 is associated with lymph node metastasis. Functional assays demonstrated that ectopic expression of SOX10 suppressed tumor cell proliferation, colony formation, migration and invasion *in vitro*. Our study thus validated that SOX10 is a functional tumor suppressor for bladder cancer.

upregulation of SOX10 in T24 cells dramatically inhibited tumor development in nude mice compared with LV-GFP control group.

SOX10 suppresses migration and invasion of bladder cancer cell lines

Next, we detected whether SOX10 regulates the invasiveness of bladder cancer cells. Wound healing assay revealed that T24 and 5637 cells infected with LV-SOX10 filled in the wound slower than LV-GFP infected cells (Figure

Epigenetic inactivation of tumor suppressor genes through promoter CpG methylation and histone modifications is a key cause of tumor initiation and progression [11]. Many SOX factors are downregulated in multiple cancers due to promoter methylation, such as SOX1 [12], SOX7 [13], SOX15 [14] and SOX17 [9]. It has been reported that promoter methylation could be the major mechanism determining whether SOX10 is silenced or activated in tumors. SOX10 is downregulated in digestive cancers with high promoter methylation status, while

SOX10 expression is upregulated in melanoma with promoter hypomethylation [14]. Here, we found that SOX10 is frequently inactivated by hypermethylation in bladder cancer cell lines, which could be restored by the DNA demethylating agent Aza. Since methylation might be a useful marker for early diagnosis or prognosis in patients with bladder cancer [15], the methylation level of SOX10 will be further confirmed in bladder cancer tissues.

SOX10 can regulate wide range of biological events by cooperating with specific partner factors to select specific target genes [16]. The recruited partner proteins could also determine transcription activities of SOX10. Several proteins have been found to interact with SOX10, such as β -catenin [8], OCT6/BRN2 [16], PAX3 [16], Olig1 [17], Egr2 [18] and so on. These interactions might contribute to SOX10-induced suppression of cell survival and metastasis. However, the biological significance of these interactions in bladder cancer needs to be explored further.

In summary, our study identifies methylation-induced SOX10 silencing plays an important role in the development of bladder cancer, which might serve as a potent target for the development of therapeutic strategies.

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Disclosure of conflict of interest

None.

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