Targeting HMGB1 inhibits bladder cancer cells bioactivity by lentivirus-mediated RNA interference

W. WANG[1], H. ZHU[3], H. ZHANG[2], L. ZHANG[2], Q. DING[1,2], H. JIANG[1,2,*]

[1]Institute of Urology, Huashan Hospital, Fudan University; [2]Department of Urology, Huashan Hospital, Fudan University; [3]Department of ICU, Huashan Hospital, Fudan University

*Correspondence: drjianghaowen@126.com

High mobility group box 1(HMGB1) has been reported to be associate with tumor clinical stage and pathological grade in bladder cancer (BC). In this study, we investigated the underlying mechanism through lentivirus-mediated HMGB1 knockdown. HMGB1 was strongly inhibited in BC cells stably transfected with shRNA against HMGB1. MTT and colony formation assays demonstrated that the down-regulation of HMGB1 attenuated the growth of BC cells in vitro. The HMGB1 knockdown (KD) group displayed an increased proportion of cells in the G0/G1 phase and higher apoptosis rates of BC cells comparing with the control (CON) group. The transwell assay revealed that the KD group cells had much lower invasive activity. To assess the influence of HMGB1 inhibition on tumorigenicity in BC cells, shRNA-HMGB1 lentivirus were injected into the tumors of xenograft models and the results showed that the tumorigenesis in mice were significantly suppressed by shRNA-HMGB1 lentivirus. Furthermore, the expression level of VEGF-C in KD group was significantly decreased comparing with the CON group. The NF-κB inhibitor PDTC reduced the expression of VEGF-C, while HMGB1, as a NF-κB agonist, enhanced the VEGF-C expression. In conclusion, our results suggested that lentiviruses delivering shRNA against HMGB1 may be a promising tool for BC therapy.

Key words: HMGB1, bladder cancer, RNA interference, proliferation

Bladder cancer (BC) is the most common genitourinary malignancy in the world the fourth most common type of cancer in men and the eighth most common in women in the United States and the 10th most common cancer in China[1, 2]. A recent study showed that 39-47% persistent nonmuscle-invasive BC patients would need a re-resection therapy [3]. About 20% re-resection T1 tumors progressed to invasive BC, and this is associated with a high rate of early metastases and poor long-term survival [4]. It was important to predict the invasive behavior of BC in clinical work, and the novel biomarkers should provide important predictive and prognostic information and be incorporated in clinical decision-making [5, 6].

High mobility group box 1(HMGB1) protein is a ubiquitous, abundant nuclear protein [7]. It had a tripartite domain organization, consisting of two DNA-binding domains, the HMG-boxes A and B, and acidic C-terminal tails of variable length [7]. HMGB1 had various biological functions within the nucleus, such as binding to DNA without sequence specificity, regulating transcription, replication, DNA repair and recombination[8]. HMGB1 could be passively released into the extracellular milieu from necrotic cells or actively secreted by various immune cells via a non-conventional specialized pathway[9]. In 1999, Wang et al. first reported that HMGB1 played an important role in mediating experimental sepsis [10]. HMGB1 could be bind to the receptor for advanced glycation end products (RAGE), and Toll-like receptors (TLR2 & 4) to trigger the inflammatory pathway [11].

Overexpression of HMGB1 had been observed in several human cancers, including hepatocellular carcinoma [12], skin squamous cell carcinoma [13], prostate carcinoma [14], gastrointestinal carcinoma [15], breast carcinoma [16] and bladder cancer (BC) [17]. Our early study also showed that HMGB1 was correlated with tumor clinical stage and pathological grade in BC[18]. It is also correlated with vascular endothelial growth factor (VEGF) expression and microvessel density (MVD) counts, suggesting HMGB1 might act as a key regulator in progression and angiogenesis of BC [18].

To further elucidate the role of HMGB1 in BC, we used lentivirus-delivered shRNA to suppress the expression of HMGB1.

Using this model system, we investigated the influence of HMGB1 shRNA on the proliferation, NF-κB/p65 and VEGF-C expression of bladder cancer cells and examined the consequent antitumor potential of HMGB1 shRNA *in vivo* and *in vitro*.

Materials and methods

Cells lines and cell culture. The 293T human renal epithelial cell, 5637 and T24 human urinary BC cell lines were purchased from American Type Culture Collection and maintained at 37°C and 5% CO_2. The 293T cell lines were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen). The 5637 and T24 BC cell lines were maintained in ATCC-formulated RPMI-1640 (Invitrogen) supplemented with 10% FBS.

Lentivirus production and cell infection. The shRNA sequence (5'-GAAGAAGATGAAGATGAAGAATTCAA-GAGATT

CTTCATCTTCATCTTCTTCTTTTTTACGCGT-3') targeting human HMGB1 gene was selected from three potential sequences (Table S1 and Fig S1). Pairs of complementary oligonucleotides for these sequences were synthesized (Genechem, Shanghai, China), annealed, and ligated into the pHBLV-U6-Puro lentiviral RNAi vector (Hanbio, Shanghai, China) linearized by *Bam*H I and *Eco*R I. The recombinant lentivirus with HMGB1-shRNA was produced by co-transfection of 293T cells with plasmids PSPAX2 and PMD2G with LipoFiter (Hanbio, Shanghai, China). Lentiviruses-containing supernatant were harvested 48 h after transfection and filtered through 0.22-μm cellulose acetate filters (Millipore, USA). Recombinant lentiviruses were concentrated by ultracentrifugation (2 h at $50,000 \times g$). These particles are hereafter referred to as Lv-shHMGB1 (specific interference construct for HMGB1), and the empty lentiviruses without HMGB1-shRNA were used as negative control (Lv-NC).

To establish stable HMGB1-konckdown cell lines, 5637 and T24 BC cells (30% confluent) were transduced with Lv-shHMGB1 at an MOI of approximately 10 in the presence of 5 μg/ml polybrene. After 24 hours, culture medium was removed and fresh medium was added to the cells. 72 hours after transduction, puromycin was added to medium at the concentration of 5 mg/ml for stable cell line selection. After antibiotic selection for 3 weeks, stable HMGB1-konckdown cells were obtained. BC cells were then divided into the following three experimental groups: the CON group (non-infected cells), the NC group (Lv-NC-infected cells), and the KD group (HMGB1-konckdown cells).

Real-time PCR and western blotting analysis. Total RNA was extracted from cells using Trizol reagent (Invitrogen) and reverse transcribed using M-MLV Reverse Transcriptase (Promega, Madison, WI) following the manufacturer's protocol. Quantitative real-time PCR reactions were prepared in a volume of 25μl containing 0.1μM primers, 10μl 2×SYBR Premix Ex Taq (Takara, Osaka, Japan), and 20-100 ng cDNA sample and assayed using a TP800 system (Takara). The primers

used were as follows: for HMGB1, 5'-ATATGGCAAAAGCG-GACAAG-3' and 5'- GCAACATCACCAATGGACAG-3; for NF-κB/p65: 5'-AAAGACTGCCGGGATGGCTTCTAT-3' and 5'-TTCCAGGTCCCGCTTCTTTACACA-3'; for β-actin, 5'-ctccatcctggcctcgctgt-3' and 5'-gctgtcaccttcaccgttcc-3'. The amplification program consisted of 95°C for 15 s and 45 cycles of denaturation at 95°C for 5s, annealing and extension at 60°C for 30 s. The relative amount of HMGB1 mRNA was calculated by the $2^{-\Delta\Delta Ct}$ method using the β-actin mRNA level for normalization[19]. All experiments were repeated at least three times.

To detect the protein level, cells were lysed using Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. The lysates were ultracentrifuged at 14,000 ×g for 30 s at 4 °C, and the supernatant (cytoplasmic extract) was collected to evaluate contents of HMGB1 and VEGF-C. The pelleted nuclei were resuspended in Complete Lysis Buffer. After 30 min at 4 °C, lysates were centrifuged at 14,000 ×g for 10min, the supernatant (nuclear extract) was collected to evaluate the content of NF-kB/p65. Endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or TATA binding protein (TBP) was used as an internal standard for cytoplasmic protein or nuclear protein respectively. Protein samples (30 μg/lane) were separated on 12% SDS-PAGE gels and transferred onto PVDF membranes (Millipore, Bedford, MA). After blocked, the membranes were incubated with rabbit anti-HMGB1 (1: 200, Abcam, Cambridge, UK), anti-NF-κB/p65 (1:1000, Abcam), anti-VEGF-C antibody (1:1000, Abcam) and mouse anti-GAPDH (1: 5000, Santa Cruz Biotechnology) and anti-TBP antibody (1: 5000, Abcam) overnight at 4°C. After washed with Tris-buffered saline/Tween-20 solution, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1: 5000, Santa Cruz Biotechnology, Santa Cruz, CA) or goat anti-mouse IgG (1: 5000, Santa Cruz Biotechnology) at room temperature for 1h. Bands were detected with enhanced chemiluminescence.

Cell proliferation and viability measurement. Cells were trypsinized and resuspended for proliferation and viability measurement. For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cells were seeded into 96-well plates ($5×10^3$ cells per well in 100μl culture medium) and incubated at 37°C. The number of viable cells was measured after 1, 2, 3, 4 and 5 days. At each time point, 10μl of 5 mg/ml MTT was added into each well, and cells were incubated for 4h. The medium was then removed, and 100μl dimethyl sulfoxide was added. The absorbance was measured at 570nm using a microplate reader (Bio-Rad).

For the cell colony formation assay, cells were seeded into dishs at a concentration of 200 cells per dish and cultured at 37°C for 14 days. Medium was replaced every 3-4 days. Cells were washed twice with PBS, fixed with paraformaldehyde, stained with Giemsa (Sigma-Aldrich, MO, USA) for 10min, and washed with ddH_2O three times. The plates were photographed with a digital camera. Each experiment was performed in triplicate and repeated three times.

Cell cycle analysis. Cells were harvested and sequentially washed with cold PBS, fixed in 70% cold ethanol, centrifuged at 400 ×g for 5min, and resuspended in PBS. Cells were then filtered through a 400-mesh membrane, stained with propidium iodide (PI) in the dark at 4°C for 30min and analyzed using flow cytometry (Becton Dickinson). Each experiment was conducted in triplicate.

Cell apoptosis assay. After cells were harvested and resuspended, FITC-Annexin V and propidium iodide (PI) were added following the instruction of annexin V-FITC apoptosis detection kit (B.D. Biosciences Pharmingen, San Jose, CA, USA). Stained cells were then quantified by flow cytometry (Becton Dickinson). Each experiment was conducted in triplicate.

Cell invasion assay. Invasion assays were performed in a six-well transwell chamber (Corning, Cambridge, USA), which contained an 8μm pore size polycarbonate membrane filter and was precoated with 100μg Matrigel (Becton–Dickinson, Bedford, USA). Cells were trypsinized and suspended in a serum-free medium containing 1% BSA at a concentration of $5×10^4$ cells/insert. Medium supplemented with 10% fetal calf serum was added to the bottom chamber. After re-culturing with 5%CO_2 at 37°C for 24h, the transwell chambers were inverted and stained with hematoxylin. Five fields were randomly selected and the number of trans-membrane cells was counted.

The concentration of VEGF-C measurement. The 5637 BC cells were treated with 10μM PDTC (NF-κB inhibitor, Beyotime, China) and cells in KD group were treated with 10μg/ml HMGB1 (NF-κB agonist, Sigma-Aldrich) for 24h. The concentrations of VEGF-C in the supernatant of CON, NC, KD, KD+PDTC and CON+HMGB1 groups were detected

using ELISA kit (R&D, MN, USA) following the manufacturer's instructions.

Animal experiments. Five-week-old male BALB/c mice were purchased from Shanghai Slac Laboratory Animal Co. Ltd (Shanghai, China). $2×10^7$ 5637 BC cells were suspended in DMEM medium and injected subcutaneously into the right flank of mice. After 10 days, mice with similar size of tumor were divided into three groups (10 mice per group) referred to as the NS (injecting 0.1ml physiological saline), Lv-NC(injecting 0.1ml $1×10^9$TU/ml Lv-NC), and Lv-HMGB1(injecting 0.1ml $1×10^9$TU/ml Lv-shHMGB1) groups. Physiological saline or lentivirus particles were injected into the xenograft tumor with above-mentioned dose every 7d. The tumor diameter was measured, and the volume (product of 0.4 × largest diameter × smallest diameter × smallest diameter) was calculated on day 10, 15, 22 and 30. Mice were photographed and humanely killed on day 30, and the tumors were dissected and weighed.

Statistical analysis. All data were expressed as mean ± SD. Statistical analysis was performed using SPSS 18.0 (SPSS, Chicago, USA). The statistical correlation of data between groups was analyzed by Student's t test. Statistical significance was accepted at $p<0.05$.

Results

Downregulation of HMGB1 expression in bladder cancer cells by lentivirus-mediated RNAi. Real-time PCR analysis showed that the mRNA expression of HMGB1 in 5637 and T24 BC cells in the KD group was significantly decreased than in the CON group and the NC group ($p<0.05$, Fig. 1A and
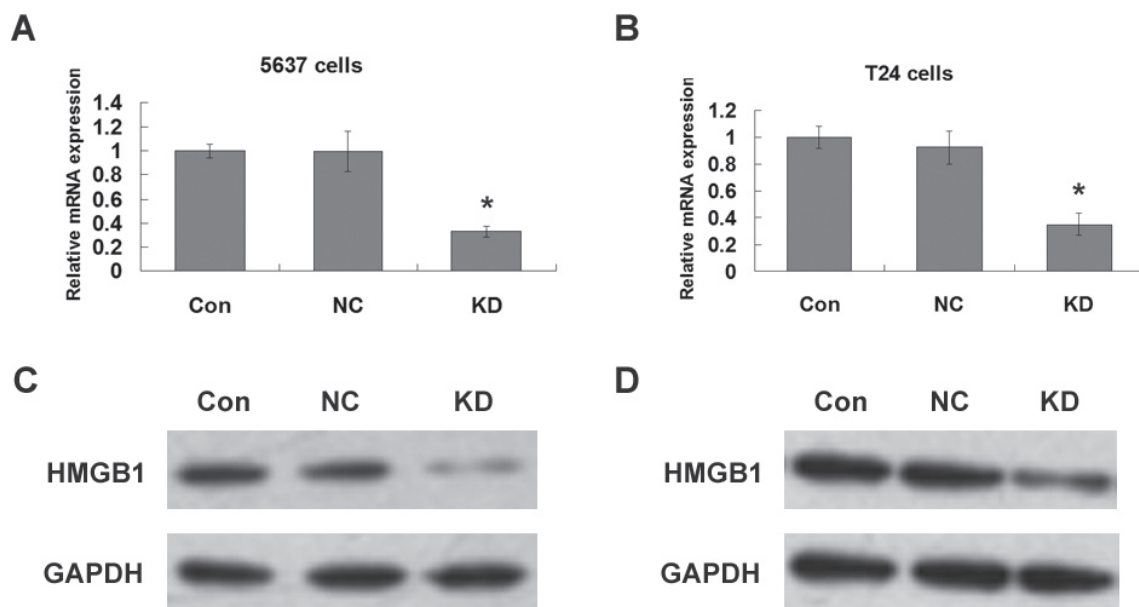
Figure 1. Lv-shHMGB1 mediated RNAi silencing significantly inhibited HMGB1 expression in BC cells. 5637 and T24 BC cells were untreated or infected with Lv-shHMGB1 or Lv-NC, and then the stably transfected cells were selected. (A) Real-time PCR analysis of HMGB1 mRNA expression. *_p_<0.05, vs CON group. (B) Immunoblot analysis of HMGB1 protein expression.

B). Immunoblot analysis showed the decreased expression of HMGB1 protein in the KD group (Fig. 1C and D). Thus, the endogenous HMGB1 has been silenced in BC cells transfected stably with shHMGB1.

Knockdown of HMGB1 inhibited the growth and invasive activity of BC cells *in vitro*. To investigate whether the downregulation of HMGB1 resulted in suppression of BC cell growth, the proliferation and viability of 5637 and T24 BC cells were assessed by MTT assay, and colony formation assay. MTT assay indicated that the KD group displayed a significant cell proliferation defect compared to the CON and NC groups ($p<0.05$, Fig. 2A). A colony formation assay revealed that the number of colonies was decreased in KD group compared to the CON and NC group ($p<0.05$, Fig. 2B).

To determine whether the downregulation of HMGB1 impacts the cell cycle and apoptosis of BC cells, all three groups of 5637 and T24 BC cells were subjected to flow cytometry analysis. The KD group of both BC cells displayed increased proportions of cells in the G0/G1 phase compared to that of the CON group and the NC group ($p<0.05$, Fig. 3A) implying the cell cycles were been blocked in G0/G1 phase. The cell apoptosis assay showed that the volume of apoptotic cells in KD group was significantly increased comparing with that in the CON and NC groups ($p<0.05$, Fig. 3B). These results partly explained how HMGB1 knockdown reduced BC cell proliferation.

The Transwell assay revealed that the KD group transfected with Lv-shHMGB1 had much lower invasive activity than the CON and NC group ($p<0.05$, Fig. 3C). Since there was no obvious differences in the number of cells in culture for 24h (Fig 2A), this suggests that suppression of HMGB1 has a direct inhibitory effect on invasion of BC cells.

Knockdown of HMGB1 inhibited the expressions of VEGF-C through NF-κB. To further explore the relationship between HMGB1 and metastasis of BC, the effect of knockdown HMGB1 on VEGF-C was detected in 5637 BC cells. As a subunit of VEGF, VEGF-C is reported to be a factor that enhanced the permeability of the lymphatic system and connected with tumor
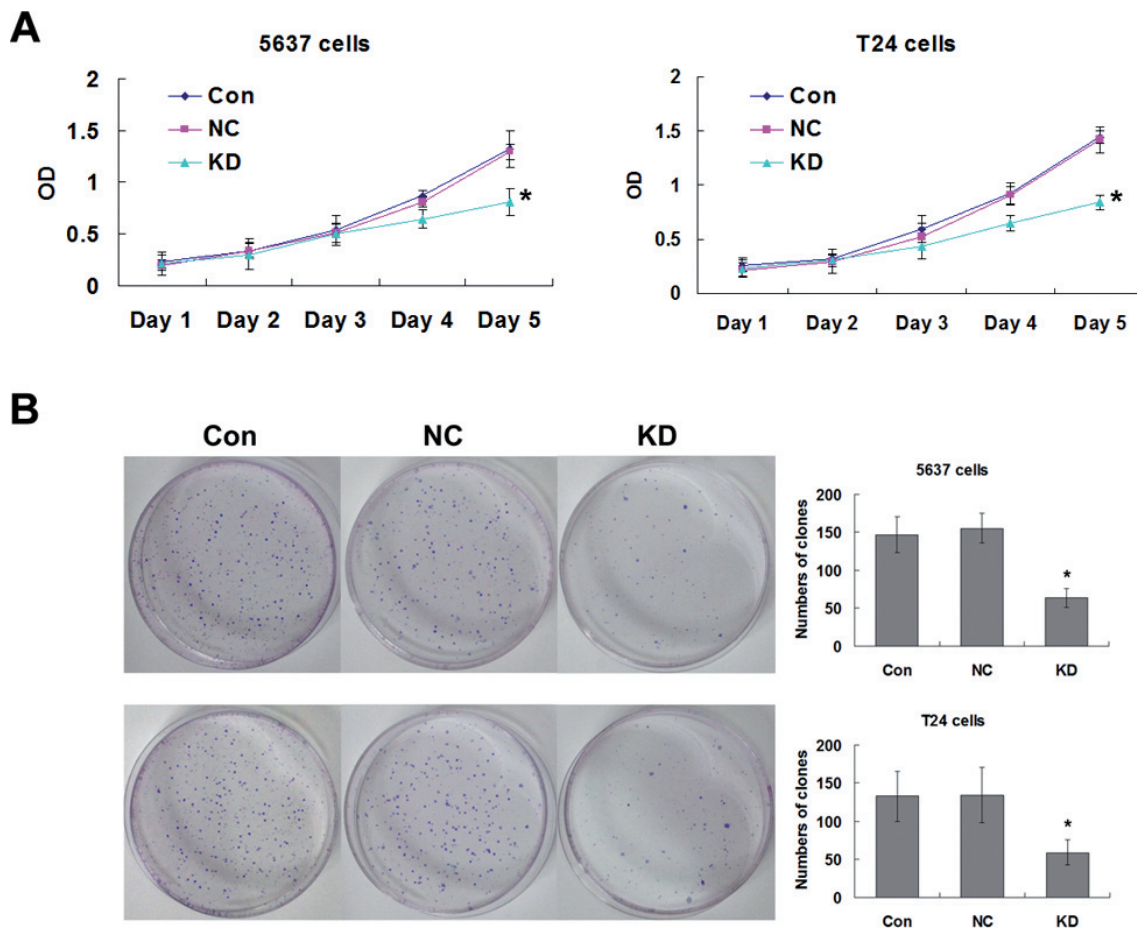

Figure 2. Knockdown of HMGB1 inhibited the growth of BC cells *in vitro*.
(A) HMGB1 down-regulation suppressed cell proliferation assessed by . MTT assay. (B) Knockdown of HMGB1 resulted in lower colony forming efficiency of BC cells. *$p<0.05$, vs CON group.
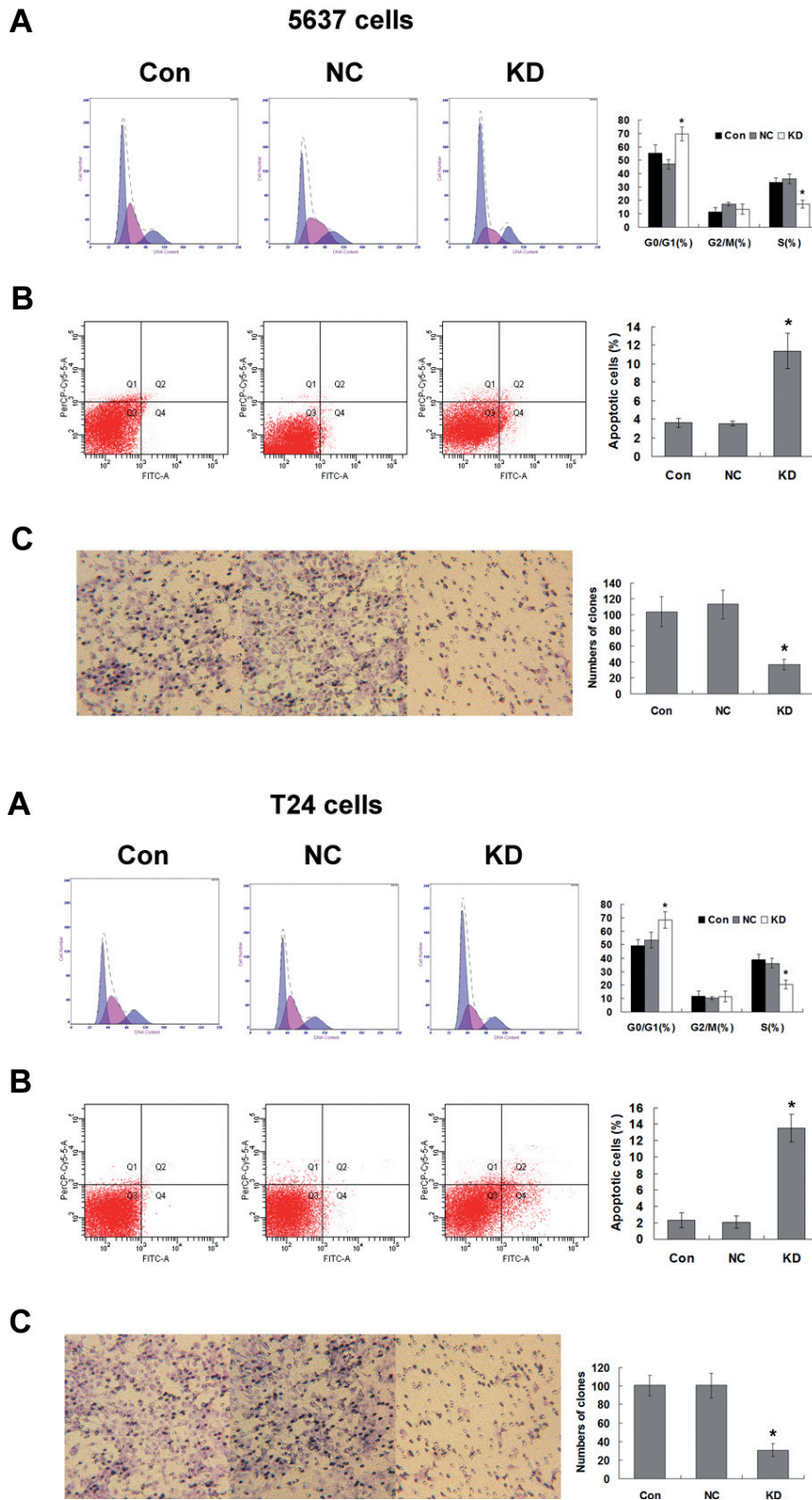
Figure 3. Knockdown of HMGB1 blocked cell cycles, increased cells apoptosis and inhibited invasive activity of BC cells *in vitro*.
(A) Cell cycles were analyzed using flow cytometry. Knockdown of HMGB1 resulted in an increase in the proportion of cells in the G0/G1 phase. (B) Cell apoptosis were determined by flow cytometry with PI/FITC-Annexin V staining. (C) Invasive cells are stained with hematoxylin and visualized by microscopy (original magnification ×200). *$p<0.05$, vs CON group.

invasion, lymph metastasis[20]. Our previous study showed that there was a significant correlation between VEGF and HMGB1 expression in BC tissue samples[18]. Moreover, increased evidences suggested that NF-κB/p65 may play an important role linking HMGB1 and VEGF[20, 21].

The Western-blot assay indicated that the expressions of VEGF-C and NF-κB/p65 were significantly decreased in the KD group compared to the CON groups ($p<0.05$, Fig. 4A and B), suggesting lentivirus-mediated knockdown of HMGB1 could inhibit the expressions of VEGF-C and NF-κB/p65. ELISA assay showed that the VEGF-C concentration was significantly decreased in the KD group (211.58±8.16 pg/ml) compared to the CON (410.77±26.51 pg/ml) groups ($p<0.05$, Fig. 4C). The NF-κB inhibitor PDTC could decrease the expression of VEGF-C (187.94±39.01 pg/ml), and HMGB1 as a NF-κB agonist could increase the expression of VEGF-C (368.54±18.79 pg/ml) significantly at the same time ($p<0.05$, Fig. 4C). It indicated that NF-κB activity was necessary for VEGF-C expression, and that HMGB1 may regulate VEGF-C expression via NF-κB signaling pathway in BC.

Injecting Lv-shHMGB1 inhibited tumorigenicity of BC cells *in vivo*. Tumors grew exponentially in all nude mice and a minimal difference was detected between mice within 10 days. Then mice were divided into three groups, the NS,

Lv-NC and Lv-HMGB1 groups, each group was given appropriate intervention. There were no significant differences on the state and weight of nude mice and no obvious abnormal changes in the tissues around tumor in KD group compared with CON group. Tumor volume of the NS group was about 840.40±146.43mm³ and weighed about 0.90±0.27g on day 30, the data of the Lv-NC group was 775.87±202.49mm³ and 0.81±0.25g. By contrast, mice in the Lv-HMGB1 group developed smaller tumors about 285.90±86.15mm³ and 0.26±0.11g ($p<0.05$, Fig. 5).

Discussion

The involvement of HMGB1 in cancer was complex. The intracellular and extracellular HMGB1 had been implicated in the response to cancer chemotherapy and also mediated of tumor formation, progression, angiogenesis, and metastasis [22]. HMGB1 might act as a sensor of DNA modification and facilitate p53 phosphorylation after exposure to genotoxic stress. It would become new component of the DNA damage signaling cascade and provide a novel promising target for chemotherapeutic intervention [23]. HMGB1 might be directly involved in cancer metastasis through promoting cell migration, modulating the adhesive properties of cells, and
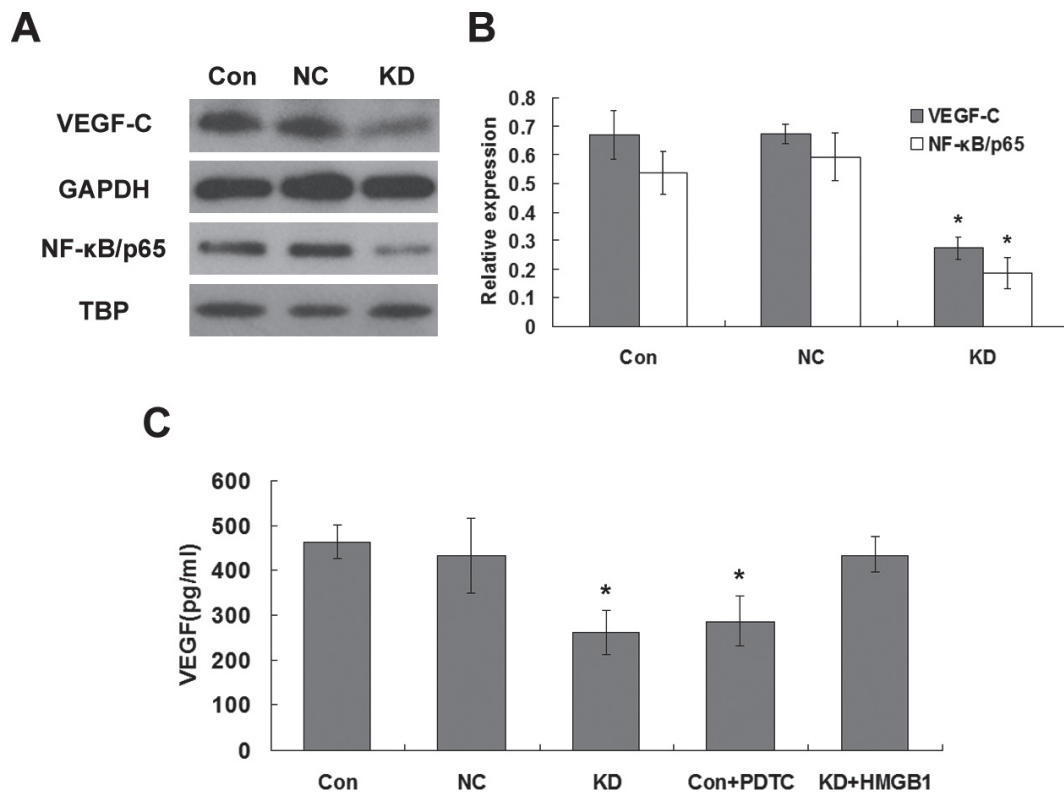
Figure 4. Lentivirus-mediated knockdown of HMGB1 inhibited the expressions of VEGF-C through NF-κB.
(A) and (B) The expressions of NF-κB/p65 and VEGF-C were quantified by Western-blot assay. (C) VEGF-C concentration was detected by ELISA assay. *$p<0.05$, vs CON group.
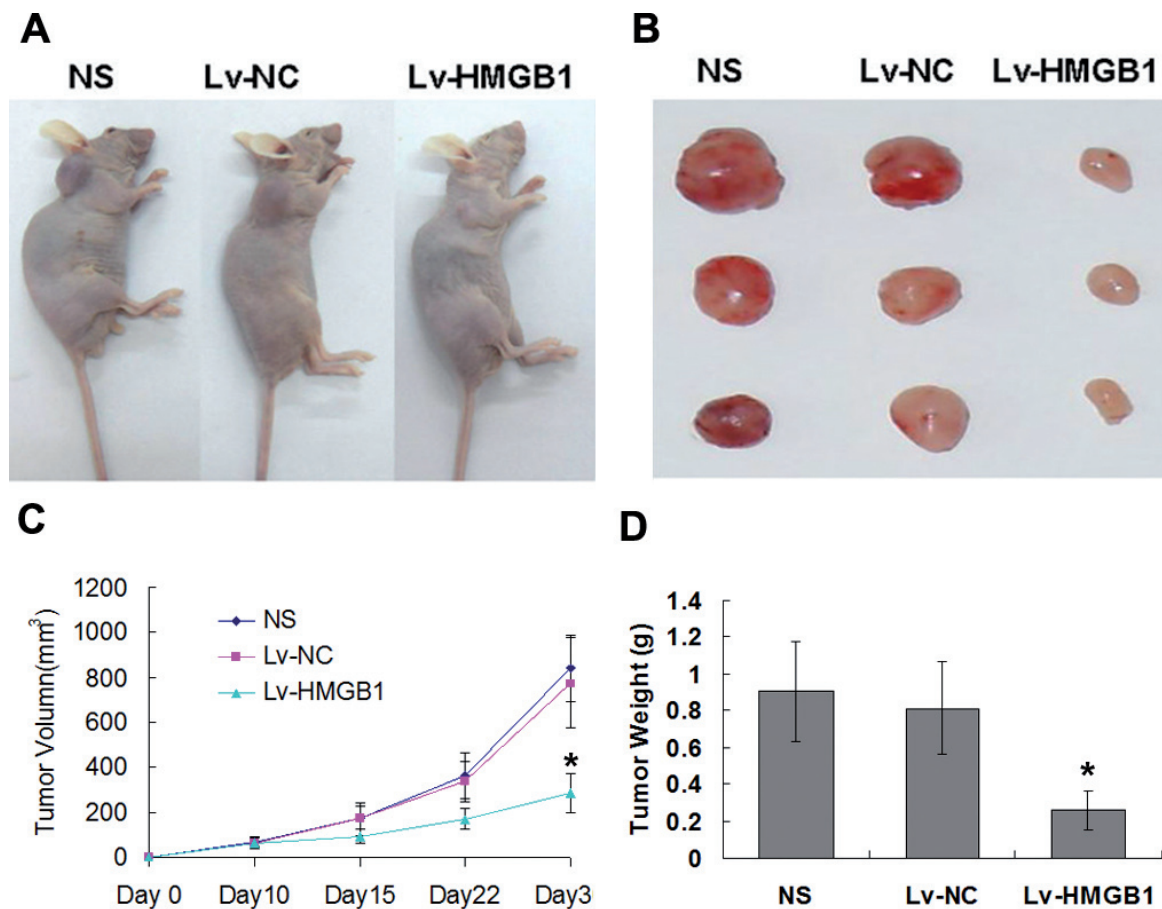
Figure 5. Injecting Lv-shHMGB1 inhibited tumorigenicity of BC cells *in vivo*
(A) and (B) Photographs of nude mice and tumors were taken 30 days after injection with BC cells. (C) Tumor volumes were recorded on day 10, 15, 22 and 30. (D) Mice were humanely killed and tumors were weighed on day 30. *$p<0.05$, vs NS group.

modifying components of the extracellular matrix [9]. HMGB1 had been recognized as a latent proangiogenic factor in cancer progression and angiogenesis, it could stimulate endothelial cell proliferation and sprouting *in vitro* and neovascularization *in vivo*, and it might due to the production of the angiogenic factors such as VEGF [24]. In this study, lentivirus-mediated HMGB1 silencing strategy was used. Knockdown of HMGB1 induced strong suppression of BC cells growth, proliferation and invasive ability *in vitro*. In addition, downregulation of HMGB1 resulted in an increase in the proportion of BC cells in the G0/G1 phase and induced cells apoptosis. Injecting sh-HMGB1 lentivirus could decrease the 5637 BC cells proliferation and made the 5637 BC cells nearly lost their tumorigenicity in nude mice models, indicating that inhibition of HMGB1 expression suppressed the tumorigenicity of 5637 BC cells *in vivo*.

Extracellular HMGB1 could bind to specific membrane receptors, including the RAGE, TLR2 and TLR4[25], and activate key cell signaling pathway NF-κB/p65 and induced cancer cell growth, apoptosis, migration, invasion, and metastasis[9, 26]. Abnormally activated NF-κB/p65 could modulate the transcription and expression of VEGF, and was important in the development, metastasis and angiogenesis of cancers[21, 27]. As a new subunit of VEGF, VEGF-C could bind to VEGFR2 and VEGFR3 to activate the MEK/ERK, PI3K/Akt and MAPK pathways and involved in angiogenesis, lymphangiogenesis and the maintenance of adult lymphatic vasculature[20, 28]. VEGF-C was overexpressed in human lung cancer, gastric cancer, colon cancer and etc. Specific inhibition of the expression of the HMGB1 could suppress NF-κB/p65 expression in hepatocellular carcinoma cells, which could reduce the expression of VEGF-C and inhibit tumor growth, proliferation, angiogenesis and invasion ability[29]. Fujioka found that inhibitied NF-κB/p65 expression could decrease the resistance of pancreatic cancer to chemotherapy[30]. Our results indicated that downregulation of HMGB1 could inhibit the expression of NF-κB/p65 and VEGF-C in 5637 BC cells and HMGB1 may regulate VEGF-C expression via the NF-κB signaling pathway. But HMGB1 signaling pathways were quite complex and many downstream

molecules of HMGB1 were not yet entirely clear. It was worth further study in the future.

In conclusion, our work provided the evidence that HMGB1 has a potentially important role in the tumorigenicity of BC. Specific lentivirus-mediated knockdown of HMGB1 significantly impacted the proliferation, cell cycle, cells apoptosis and invasive ability of BC cells. Injecting lentivirus delivering shRNA against HMGB1 could decrease the BC cells proliferation and made the BC cells nearly lost their tumorigenicity in nude mice models. Furthermore, HMGB1 could regulate VEGF-C expression via the NF-κB signaling pathway possibly. This novel strategy may be a promising tool for BC therapy.

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Supplementary Information

Targeting HMGB1 inhibits bladder cancer cells bioactivity by lentivirus-mediated RNA interference

W. WANG[1], H. ZHU[3], H. ZHANG[2], L. ZHANG[2], Q. DING[1,2], H. JIANG[1,2,*]

[1]Institute of Urology, Huashan Hospital, Fudan University; [2]Department of Urology, Huashan Hospital, Fudan University; [3]Department of ICU, Huashan Hospital, Fudan University
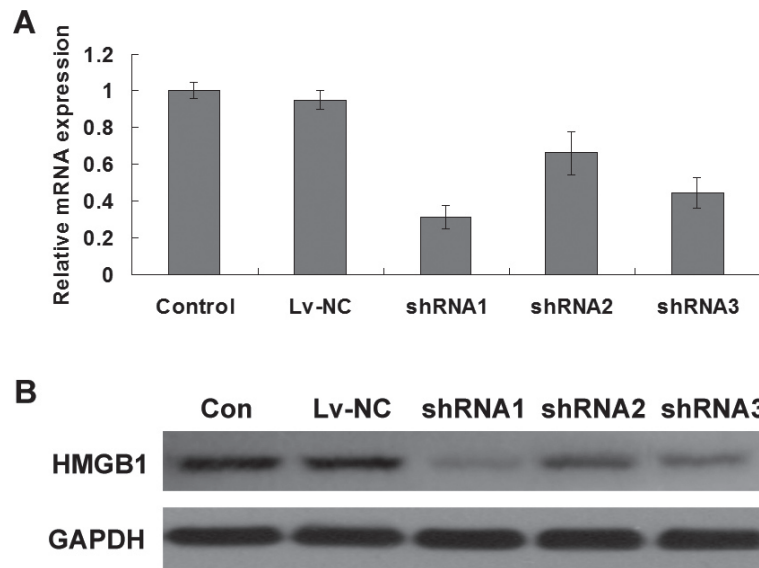
*Correspondence: drjianghaowen@126.com

Figure S1. Interference efficiency of three potential shRNA sequences.
5637 BC cells were untreated or infected with Lv-shHMGB1 (shRNA1, 2 or 3) or Lv-NC. (A) Real-time PCR analysis of HMGB1 mRNA expression.(B) Immunoblot analysis of HMGB1 protein expression.

Table S1 The designed shRNA sequences targeting human HMGB1 gene

	siRNA sequences	shRNA sequences
1	5'-GAAGAAGATGAAGATGAAGAA-3'	5'-GAAGAAGATGAAGATGAAGAATTCAAGAGATTCTTCATCTTCATCTTCTTCTTTTTTACGCGT-3'
2	5'-TGGGCAAAGGAGATCCTAAGA-3'	5'-TGGGCAAAGGAGATCCTAAGATTCAAGAGATCTTAGGATCTCCTTTGCCCATTTTTTACGCGT-3'
3	5'-CGGGAGGAGCATAAGAAGAAG-3'	5'-CGGGAGGAGCATAAGAAGAAGTTCAAGAGACTTCTTCTTATGCTCCTCCCGTTTTTTACGCGT-3'