

# LASS2 inhibits growth and invasion of bladder cancer by regulating ATPase activity

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**Abstract.** *Homo sapiens* longevity assurance homolog 2 of yeast LAG1 (LASS2) is a novel suppressor of human cancer metastasis, and downregulation of LASS2 has been associated with a poor prognosis in patients with bladder cancer (BC). However, the molecular mechanism underlying LASS2-mediated inhibition of tumor invasion and metastasis in BC remains unclear. LASS2 has been reported to directly bind to subunit C of vacuolar H<sup>+</sup>-ATPase (V-ATPase) in various types of cancer, suggesting that LASS2 may inhibit cancer invasion and metastasis by regulating the function of V-ATPase. The present study investigated the effect of LASS2-specific small interfering (si)RNA on the invasion and metastasis of the RT4 human BC cell line, which has a low metastatic potential, and its functional interaction with V-ATPase. Silencing of LASS2 in RT4 cells was able to increase V-ATPase activity, the extracellular hydrogen ion concentration and, in turn, the activation of secreted matrix metalloproteinase (MMP)-2 and MMP-9, which occurred simultaneously with enhanced cell proliferation, cell survival and cell invasion *in vitro*, as well as acceleration of BC growth *in vivo*. In this process, it was found that siRNA-LASS2 treatment was able to suppress cell apoptosis induced by doxorubicin. These findings suggest that silencing of LASS2 may enhance the growth, invasion and metastasis of BC by regulating ATPase activity.

## Introduction

Bladder cancer (BC) is one of the leading causes of cancer-associated mortality worldwide, in which 90% of cases exhibit a transitional cell histology; its incidence is second only to prostate cancer as a malignancy of the genitourinary

tract and it is the second most common cause of genitourinary cancer-related mortality (1). Although radical cystectomy with urinary tract reconstruction is considered the standard treatment for BC, the mortality rate from invasive urothelial cell carcinoma is ~50%, and there is a significant decrease in quality-of-life following radical surgery (2). Every year, >200,000 people succumb to BC due to cancer metastasis (3). Therefore, the investigation of the mechanism underlying BC metastasis, and the search for genes associated with BC metastasis and therapeutic targets, is necessary.

Numerous genes may be associated with the invasive capability of BC cells. For example, upregulation of *Homo sapiens* longevity assurance homolog 2 of yeast LAG1 (LASS2) was shown to correlate with an increased invasiveness of BC cells (4). LASS2, which is also known as tumor metastasis suppressor gene 1 (TMSG1; GenBank accession no. AF189062), and is a novel gene isolated from a human liver cDNA library in the laboratory of Shanghai Medical College, Fudan University (Shanghai, China), is a human homolog of the yeast longevity assurance gene LAG1 (*Saccharomyces cerevisiae* longevity assurance gene) (5). Several studies have correlated LASS2 with the extent of invasion and recurrence in prostate (6-8), liver (9) and breast (10,11) carcinomas. Furthermore, previous studies have reported that LASS2 is able to interact with subunit C of vacuolar H<sup>+</sup>-ATPase (V-ATPase) (11,12) to regulate V-ATPase activity and the extracellular H<sup>+</sup> concentration, and in turn activate secreted matrix metalloproteinases (MMPs) 2 and 9, leading to inhibition of cell proliferation, cell survival, invasion and metastasis (10,11). However, the mechanism of LASS2-mediated inhibition of tumor invasion and metastasis in BC has yet to be investigated. In our previous study, LASS2-negative BC was associated with a poor clinical prognosis, and the expression of LASS2 was significantly correlated with clinical stage, tumor depth and recurrence (13). In addition, different LASS2 expression levels were observed among the BIU-87, T24, EJ and EJ-M3 human BC cell lines from patients with poorly-, moderately- or well-differentiated disease, which indicated that LASS2 expression may be correlated with the development and progression of human BC (14).

In the present study, small interfering RNAs (siRNAs) targeting the LASS2/TMSG1 gene were transfected into the RT4 human BC cell line, which has a low metastatic potential (15), in order to further evaluate the inhibitory effect of LASS2 on the

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growth, invasion and metastasis of BC cells. The experiments were designed to elucidate the potential mechanisms underlying the effects of LASS2 on the inhibition of cancer metastasis by investigating the activities of V-ATPase, MMP-2 and MMP-9, as well as the apoptosis of the transfected cells.

## Materials and methods

**Cell culture and transfection.** The RT4 and T24 human BC cell lines (The Second Affiliated Hospital of Kunming Medical University, Yunnan Institute of Urology, Kunming, China) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Invitrogen RPMI-1640 medium, PBS, Opti-MEM I, Lipofectamine 2000 and glutamine were purchased from Thermo Fisher Scientific, Inc.

Two siRNA sequences targeting LASS2 [National Center for Biotechnology Information (NCBI) accession nos. NM013384, NM181747 and NM022075] were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China): siRNA-1, 5'-GGC UAUUACUUCUCAAUUTT-3' and siRNA-2, 5'-CAGUAU UGGUACUACAUGATT-3'. Unspecific control siRNA (si-NC) was also obtained from Guangzhou RiboBio Co., Ltd. The siRNAs were transfected into the RT4 cell line using Lipofectamine 2000, according to the manufacturer's protocol. siRNA was used for the *in vitro* experiments, and shRNA was used for cell selection and then for the Xenograft model.

**sh-LASS2 cell line selection.** sh-LASS2 cell line selection was performed as described previously by Xu *et al* (16). Briefly, two complementary oligonucleotides (5'-CACCGAAGAAAGTTT **GGGAGGGATAATTCAAGACGTATCCCTCCCAA**CTTTCTTCTTTTTTG-3' and 5'-AGCTCAAAAAAGAAGAAA **GTTTGGGAGGGATACGTCTTGAATATCCCTCCCAA**CTTTCTTC-3'; the 21-nucleotide sense or antisense strand is in bold letters and the stem loop sequences are in italics) were synthesized (Sangon Biotech, Co., Ltd., Shanghai, China), annealed to generate dsDNAs and ligated into the linearized empty vector pSilencer2.1-U6 (Ambion; Thermo Fisher Scientific, Inc.). A random sense sequence (AAGCTCAGGTCACATCTCTGC) was used as a negative control. The constructed plasmids were verified by direct sequencing (Sangon Biotech, Co., Ltd.). Stable transfection of RT4 cells was performed using Lipofectamine 2000 according to the manufacturer's protocol. Briefly, 1x10<sup>5</sup> cells was seeded and transfected using 2 µg DNA mixed with 5 µl Lipofectamine in 1 ml Opti-MEM I. The cells were incubated for 24 h at 37°C, and then 1 ml medium containing 20% RPMI-1640 medium was added into each well. The cells were cultured and maintained in medium containing 400 mg/ml G418 (Invitrogen; Thermo Fisher Scientific, Inc.) for at least 2 weeks until the nontransfected RT4 cells cultured in the controlled wells were all killed. Subsequently, the expression level of LASS2 was determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis.

**RNA extraction and RT-qPCR.** Cells were harvested, centrifuged for 10 min at 600 x g at 4°C, and washed with PBS. Total RNA

was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RNA was quantified at 260 nm in a NanoDrop spectrophotometer, with an optical density 260/280 ratio of 1.7/2.0 for all samples. First-strand cDNA was synthesized in a volume of 20 µl using 1 µg total RNA and a High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). Target sequences were obtained from the NCBI GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>). qPCR was performed using a LightCycler® 480 System (Roche Diagnostics, Basel, Switzerland) and the qPCR Master-Mix (Kapa Biosystems, Inc., Wilmington, MA, USA), according to the manufacturers' protocols. To quantify target mRNA levels, Applied Biosystems LASS2 Gene Expression assays were purchased from Thermo Fisher Scientific, Inc. The primer sequences were as follows: LASS2 forward, 5'-GCCTTGCTCTTCCTCATCGTTC-3' and reverse, 5'-TGCTTGCCACTGGTCAGGTAGA-3'; and GAPDH (housekeeping gene) forward, 5'-GGTCTCCTCTGACTTCAACA-3' and reverse, 5'-GAGGGTCTCTCTCTTCCT-3'. All PCRs were run in duplicate and were performed for 40 cycles (95°C for 15 sec and 60°C for 30 sec). Relative expression levels were determined following normalization to GAPDH using the 2<sup>-ΔΔC<sub>q</sub></sup> method (17). All tests were performed in triplicate.

**Western blot analysis.** For the western blot analysis, the cells were harvested in lysis buffer containing 50 mM Tris (pH 8.0), 2% SDS, 1 mM EDTA and 150 mM NaCl. The homogenate was centrifuged for 10 min at 14,000 x g at 4°C and the supernatant was used for protein determination. The protein content was measured using the Lowry method (Bio-Rad DC Protein Assay; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell lysate proteins (30 µg) were separated by 10% SDS-PAGE, transferred onto Trans-Blot Transfer Medium Membranes (Bio-Rad Laboratories, Inc.) and blocked with 5% skimmed milk for 1 h at room temperature. The membranes were incubated with anti-LASS2 (catalog no. sc-390745; 1:500 dilution), anti-β-actin (sc-47778; 1:1,000 dilution), anti-caspase-3 (catalog no. sc-7272; 1:500 dilution) and anti-poly(ADP-ribose) polymerase (PARP) (catalog no. sc-7150; 1:500 dilution) antibodies from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) at 4°C overnight. The membranes were then incubated with goat anti-mouse immunoglobulin G-horseradish peroxidase-conjugated secondary antibodies (catalog no. sc-2005; 1:2,000 dilution) for 2 h at room temperature. Western blots were developed using the enhanced chemiluminescence technique (GE Healthcare Life Sciences, Chalfont, UK) and band intensity was quantified using Quantity One software (Bio-Rad Laboratories, Inc.).

**Cell viability assay.** MTT assays (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) were performed to evaluate cell viability, according to the manufacturer's protocol. Briefly, the si-LASS2-transfected RT4 cells and the controls were seeded into 96-well plates at a density of 1.0x10<sup>3</sup> cells/well in triplicate. MTT solution (20 µl, 5 mg/ml; Sigma-Aldrich; Merck Millipore) was added to each well and 150 µl dimethyl sulfoxide was added 4 h later to dissolve the crystals. Subsequently, the absorbance of the cells was measured at 490 nm using an ELISA reader.

**Cell migration assay.** Cell migration was measured by wound healing (scratch) assays. For the scratch assay, cells were

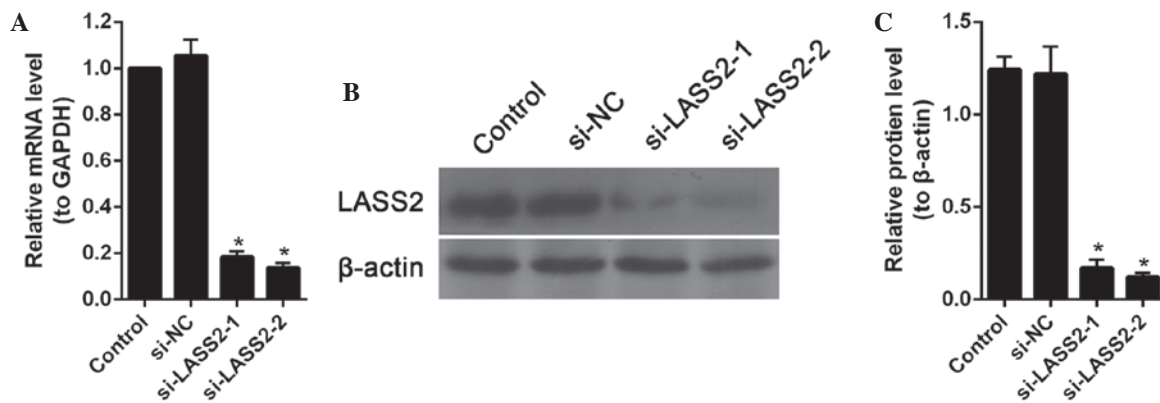


Figure 1. Effect of siRNA on the expression of LASS2 in bladder cancer cells. (A) Quantitative analysis of LASS2 mRNA was performed by reverse transcription-quantitative polymerase chain reaction. (B) The expression of LASS2 protein in RT4 cells was assessed by western blotting. (C) Semiquantitative analysis of the western blot result. Data are presented as the mean  $\pm$  standard deviation (n=3). \*P<0.05 vs. the control group. Control, group without transfection; si-NC, group transfected with negative control siRNA; siRNA, small interfering RNA; LASS2, longevity assurance homolog 2 of yeast LAG1.

seeded into 6-well plates in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin, at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were grown to confluence and a scratch was created using a sterile pipette tip. The ability of the cells to migrate into the scratch area was assessed under a Leica DM6000B microscope (Leica Microsystems GmbH, Wetzlar, Germany) 24 h after the creation of the wound area.

**Cell matrigel invasion assay.** The invasive ability of RT4 and T24 cells was evaluated using a Matrigel invasion assay. BD BioCoat Matrigel Invasion Chambers with 8-mm pore polyethylene terephthalate membranes (BD Biosciences, Franklin Lakes, NJ, USA) for 24-well plates were prepared by hydrating for 2 h at 37°C. A total of  $2 \times 10^5$  cells in 0.2 ml were seeded into each insert. After 12-h cultures, the invasion chamber was removed, the medium in the top wells was aspirated and the cells on the upper surface of the membranes were removed using cotton swabs. Invading cells attached to the lower side of the membrane were removed by flushing with a pipette, after which migrating cells present in the bottom chambers were stained with crystal violet and then counted under a light microscope. Fluorescence intensities were determined and plotted on a standard histogram and the number of invading cells was calculated. All determinations were performed in triplicate. Data are expressed as the number of invaded cells.

**Activity of V-ATPase.** To measure lysosomal V-ATPase activity, an acridine orange (Invitrogen; Thermo Fisher Scientific, Inc.) uptake assay was performed using isolated lysosomes, as previously described (18). Furthermore, the extracellular pH (pHe) of the cells was measured using the fluorescent pH indicator, 2',7'-bis-(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF) (Molecular Probes; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Briefly, three groups of RT4 cells in six-well plates were cultured, then transiently transfected with si-LASS2. After 24 h,  $1 \times 10^4$  cells were seeded into 96-well plates, cultured at 37°C in 5% CO<sub>2</sub> for 24 h, and then the pHe was detected using a 96-well fluorospectrophotometer following staining with 1  $\mu$ M BCECF.

**MMP zymography.** The zymography assay was performed in 10% SDS polyacrylamide gels containing 0.1 mg/ml gelatin. Protein sample (20  $\mu$ g) was loaded into each lane of the gel. After electrophoresis, the gel was washed twice with 2.5% Triton X-100 for 1 h at room temperature to remove SDS, and then incubated at 37°C overnight in reaction buffer (Applygen Technologies, Inc., Beijing, China). After staining with Coomassie brilliant blue, MMP-2 and MMP-9 expression was identified as clear zones against the blue background.

**Cell apoptosis analysis.** After treating with si-LASS2 or 20  $\mu$ g/ml doxorubicin (Dox; Sigma-Aldrich; Merck Millipore) for 48 h, RT4 cells were harvested and stained with Annexin V-fluorescein isothiocyanate and propidium iodide (Invitrogen; Thermo Fisher Scientific, Inc.) prior to fluorescence-activated cell sorting (BD Biosciences) to analyze apoptosis, according to the manufacturers' protocols.

**Xenograft model and treatments.** A total of 15 BALB/c male nude mice (4-5 weeks old; Beijing Vital River Laboratory, Beijing, China) were adapted to the conditions for 3 days prior to starting the experiment. The room temperature was 20-25°C, with a relative humidity of 40-70%. Drinking water, bedding and feeding cages were autoclaved prior to being provided to then mice. The mice were randomly divided into 3 groups (5 per group) as follows: The control group, the shR-NC group and the shR-LASS2 group. For the *in vivo* assays,  $1 \times 10^6$  RT4 cells and RT4-sh-LASS2 cells were injected into the flank of athymic nude mice (5 mice/group) and tumor dimensions were measured using a caliper once per week. The tumor volumes were calculated using the following formula: Tumor volume (mm<sup>3</sup>) = length (mm)  $\times$  width (mm)  $\times$  width (mm)  $\times$  0.52. At week 6, all mice were anesthetized with pentobarbital (50 mg/kg) and euthanized by cervical dislocation. Tumor tissues were harvested, fixed in 4% formalin, and subsequently dehydrated and embedded in paraffin for hematoxylin and eosin staining. All protocols for treating animals were approved by the Animal Use Committee of Kunming Medical University (Kunming, China).



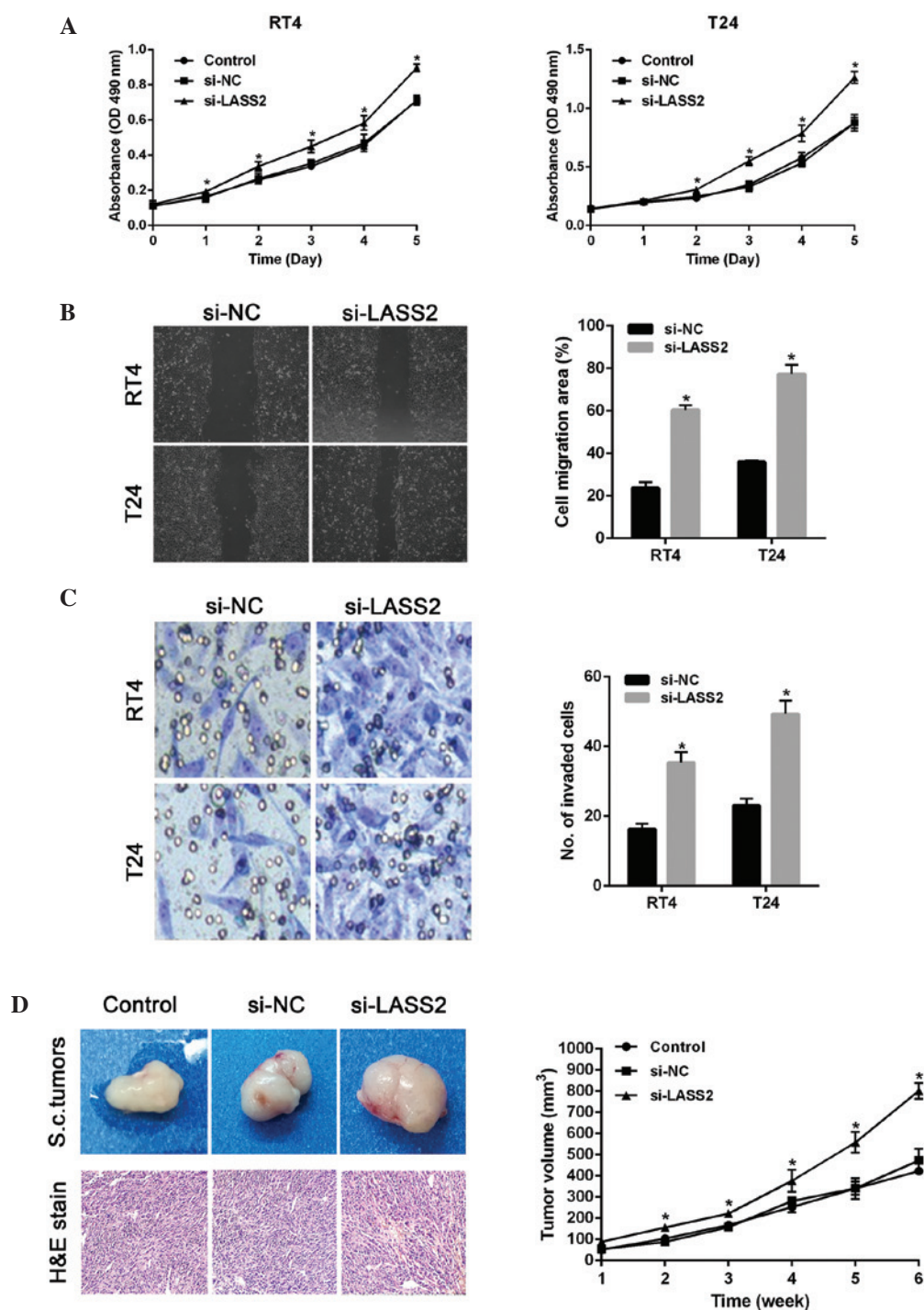


Figure 2. si-LASS2 exhibits cancer promoting effects *in vitro* and *in vivo*. (A) MTT assays were used to assess the viability of RT4 and T24 cells transfected with si-LASS2 or si-NC. (B) Cell migration was measured using scratch assays (x40 magnification). (C) Matrigel invasion assays were used to evaluate the invasion activity of bladder cancer cells transfected with si-LASS2 or si-NC (x100 magnification). (D) Tumor outgrowth in RT4 xenograft mice (x40 magnification). Top images, representative tumors from the three groups after 6 weeks; bottom images, dissected tumors stained with H&E. Data are presented as the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$  vs. si-NC group. si-LASS2, small interfering RNA targeting longevity assurance homolog 2 of yeast LAG1; si-NC, unspecific control small interfering RNA; H&E, hematoxylin & eosin.

**Statistical analysis.** Statistical analysis was performed using SPSS 19.0 software (IBM SPSS, Armonk, NY, USA). All assays were repeated three times, data are expressed as the mean  $\pm$  standard deviation and  $P < 0.05$  was considered to indicate a statistically significant difference. The significance of the difference was determined using Student's *t*-tests and  $\chi^2$  tests. All tests were two-tailed for unpaired data.

## Results

**Downregulation of LASS2 by RNA interference.** RT4 cells, which are BC cells with a low metastatic potential (15), were transfected with siRNA targeting LASS2 or with si-NC. After transfection for 48 h, the expression levels of LASS2 in the transfected cells were measured using RT-qPCR and western

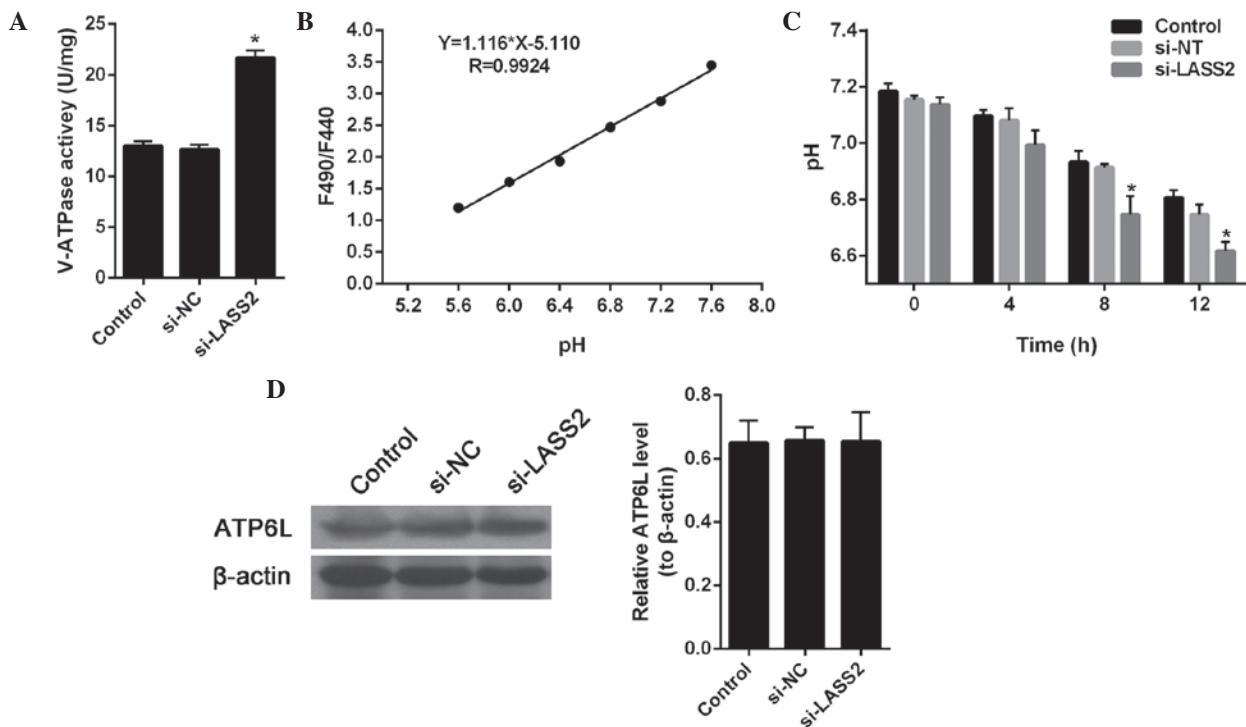


Figure 3. Activity of V-ATPase in si-LASS2 cells. (A) V-ATPase activity in RT4 cells was assessed using the acridine orange uptake assay. (B) Standard curve for the pH assay. (C) pH values significantly decreased as a result of increased proton secretion in si-LASS2 cells compared with the control or si-NC cells. (D) si-LASS2 treatment did not alter the expression levels of ATP6L in bladder cancer cells, as determined by western blotting. Data are presented as the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$  vs. control group. V-ATPase, vacuolar  $H^+$  ATPase; si-LASS2, small interfering RNA targeting longevity assurance homolog 2 of yeast LAG1; si-NC, unspecific control small interfering RNA; pH, extracellular pH.

blot analysis. Compared with the control cells and si-NC-transfected cells, the levels of LASS2 mRNA and protein were significantly downregulated in the cells transfected with siRNA targeting LASS2 ( $P < 0.001$ ; Fig. 1). According to these results, si-LASS2-2, which showed the greatest inhibition on LASS2 expression, was used for further analyses.

**siRNA targeting LASS2 promotes cancer effects in vitro and in vivo.** To investigate the cancer promoting potential of si-LASS2, two BC cell lines, RT4 and T24, were transfected with si-LASS2. Cell viability was examined using MTT assays. As shown in Fig. 2A, significant increases in cell viability were observed following transfection with si-LASS2, as compared with the si-NC-transfected or untransfected cells. Subsequently, the impact of LASS2 on the migration and invasion of BC cells was investigated. The migration of si-LASS2-transfected cells was significantly increased for both cell lines, as compared with the si-NC-transfected cells ( $P < 0.001$ ; Fig. 2B). Furthermore, Transwell assays indicated that the number of invading cells was significantly increased for si-LASS2-transfected cells compared with si-NC-transfected cells ( $P < 0.001$ ; Fig. 2C). These results suggest that si-LASS2 exerts cancer promoting effects *in vitro*.

Nude mice were subcutaneously injected with RT4-sh-LASS2 cells, RT4-sh-control cells or untransfected cells. After 6 weeks, the volumes of tumor xenografts from the RT4-si-LASS2 group were significantly increased compared with the control group ( $P < 0.001$ ; Fig. 2D).

**LASS2 regulates V-ATPase activity and the extracellular pH.** A previous study showed that LASS2 is able to interact with

the C-subunit of V-ATPase and inhibit tumor growth, invasion and metastasis (9). Therefore, in the present study, the effect of si-LASS2 on V-ATPase activity and the  $H^+$  potential of RT4 cells was investigated. The results showed that si-LASS2 significantly upregulated V-ATPase activity in RT4 cells, as compared with the untreated cells ( $P < 0.001$ ; Fig. 3A), and the extracellular  $H^+$  concentration of the si-LASS2 cells was markedly increased compared with the untransfected cells and si-NC-transfected cells (Fig. 3B and C). These results suggest that si-LASS2 may promote cancer activity by regulating the activity of the V-ATPase proton pump.

In addition, the expression levels of ATP6L in RT4 cells transfected with si-LASS2 were detected by western blotting (Fig. 3D). The immunoblot results showed no change in expression, indicating that LASS2-mediated regulation of ATP6L does not occur via regulation of its expression.

**Effect of LASS2 on BC cell apoptosis.** It has previously been demonstrated that LASS2 is able to reduce the ability of V-ATPase to pump  $H^+$  out of the cell, resulting in a raise in the intracellular pH (pHi) and induction of cell apoptosis via the mitochondrial pathway (9). In order to verify whether this process occurs in BC, the apoptosis of RT4 cells was further evaluated by flow cytometric analysis. After 24 h, 20  $\mu$ g/ml Dox significantly induced the apoptosis of RT4 cells ( $P < 0.001$ ), which could be significantly inhibited by treatment with si-LASS2 ( $P = 0.0093$ ). There was no significant difference between the RT4 cells transfected with si-NC and si-LASS2 without Dox treatment ( $P = 0.4357$ ; Fig. 4A and B).

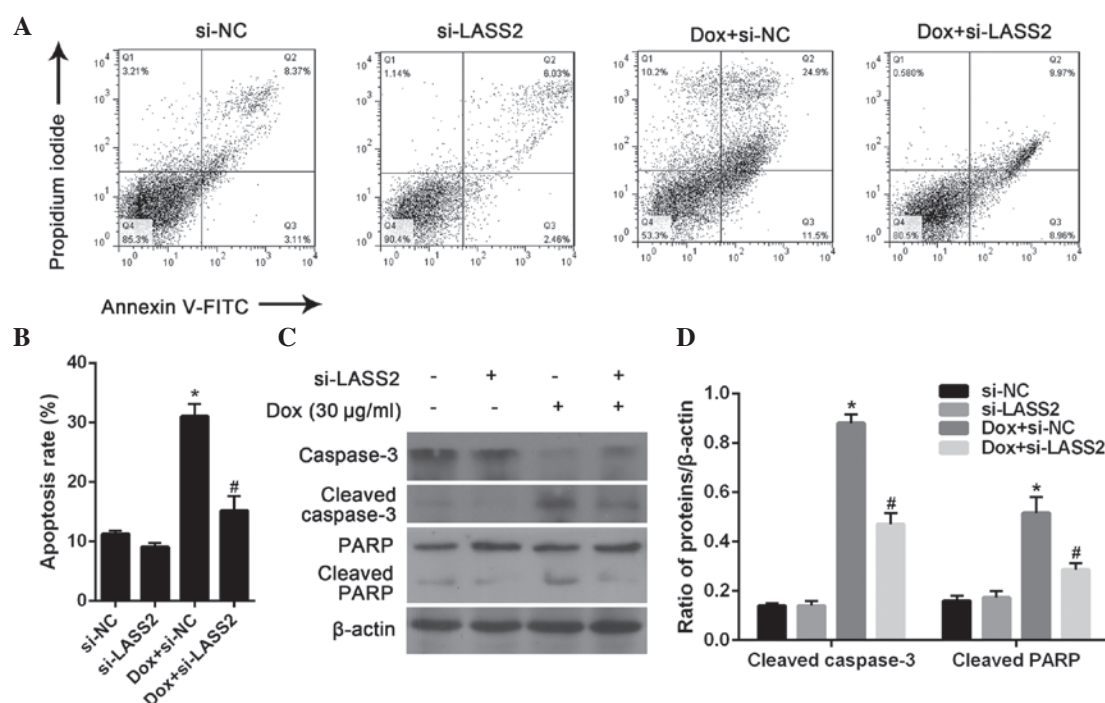


Figure 4. si-LASS2 suppresses Dox-induced bladder cancer cell apoptosis. (A) RT4 cells were transfected with si-NC or si-LASS2 in the presence or absence of Dox for 48 h, and then stained with the Annexin V-propidium iodide kit. (B) Histogram showing apoptosis induction (% total) in the different groups. Data are presented as the mean  $\pm$  SD of three independent experiments. \* $P$ <0.05 vs. the si-NC group; # $P$ <0.05 vs. the Dox+si-NC group. (C) RT4 cells showed activation of caspase-3 and PARP proteins. Whole-cell extracts were prepared and analyzed by western blotting using antibodies against caspase-3 and PARP.  $\beta$ -actin was used as an internal control to show equal loading of proteins. (D) Quantitation of caspase-3 and PARP protein levels. Data are presented as the mean  $\pm$  SD of three independent experiments. \* $P$ <0.05 vs. the si-NC group; # $P$ <0.05 vs. the Dox+si-NC group. si-LASS2, small interfering RNA targeting longevity assurance homolog 2 of yeast LAG1; si-NC, unspecific control small interfering RNA; Dox, doxorubicin; SD, standard deviation; PARP, poly(ADP-ribose) polymerase; FITC, fluorescein isothiocyanate.

Subsequently, the expression of apoptosis marker proteins in RT4 cells was analyzed by western blotting. The results showed that si-LASS2 alone had little effect on the activation of caspase-3 and PARP cleavage, while Dox treatment was highly effective. It was also observed that Dox-induced changes in caspase-3 and PARP activation were significantly suppressed following treatment with si-LASS2 for 24 h (Fig. 4C and D). These results suggest that si-LASS2 suppresses the apoptosis of RT4 cells.

*LASS2-mediated suppression of V-ATPase occurs concomitantly with the inhibition of gelatinase.* According to previous reports, MMP-2 and MMP-9 are closely related to cancer metastasis (19). Therefore, the present study assessed the activities of MMP-2 and MMP-9 using gelatin zymography. The results the gelatinase activity assay showed that the activities of MMP-2 and MMP-9 were significantly increased in the si-LASS2-transfected cells compared with the untransfected cells ( $P=0.004$  and  $P=0.018$ , respectively) and si-NC-transfected cells ( $P=0.006$  and  $P=0.025$ , respectively) (Fig. 5A and B).

## Discussion

LASS2 is a novel gene and has previously been shown to function as a tumor metastasis suppressor gene, with an important role in prostate, liver and breast carcinomas (20). Our previous study demonstrated that LASS2-negative BC was associated

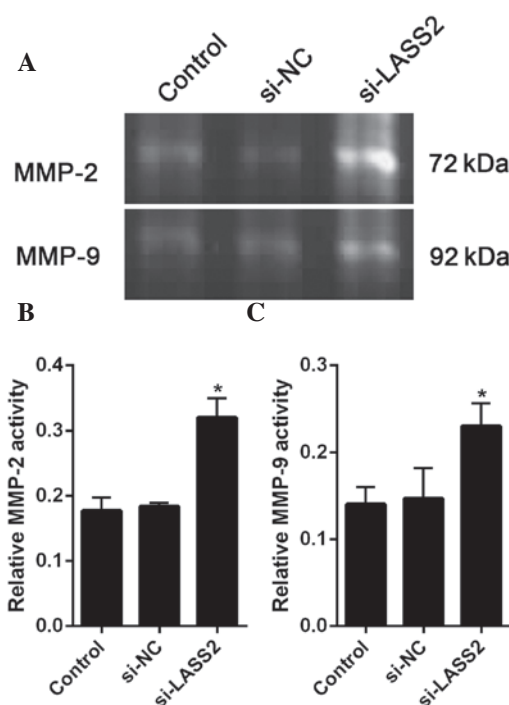


Figure 5. Gelatinase activities of MMP-2 and MMP-9 in RT4 cells. (A) Zymography was performed using a 0.1% gelatin gel, followed by Coomassie blue staining. Bar diagrams represent fold-changes in (B) MMP-2 and (C) MMP-9 activities, respectively. Values are presented as the mean  $\pm$  standard deviation from three independent experiments. \* $P$ <0.05 vs. si-NC group. MMP, matrix metalloproteinase; si-LASS2, small interfering RNA targeting longevity assurance homolog 2 of yeast LAG1; si-NC, unspecific control small interfering RNA.



with a poor clinical prognosis, and the expression of LASS2 was significantly correlated with clinical stage, depth of tumor invasion and recurrence (14). In the present study, the mechanism underlying LASS2-mediated regulation of tumor cell growth was investigated. First, BC cell lines were transfected with siRNA-targeting LASS2, and it was observed that si-LASS2 was able to promote the growth, invasion and metastasis of BC *in vivo* and *in vitro*.

Xu *et al* (16) reported that LASS2 could interact with ATP6L, reduce the ability of the V-ATPase proton pump to transport H<sup>+</sup> out of the cell, raise the H<sup>+</sup> concentration in the cell and induce cell apoptosis via the mitochondrial pathway, thereby inhibiting the growth of tumor cells. In addition, the reduction in the extracellular H<sup>+</sup> concentration was shown to reduce the activation of MMP-2 and MMP-9, thereby inhibiting tumor invasion and metastasis (16). In the present study, similar results were observed, although regulation of LASS2 did not alter the expression levels of ATP6L. Furthermore, the present study indirectly confirmed that LASS2 is able to bind to ATP6L, which has been reported in previous studies (6,10,16,20). The authors of the present study hypothesized that, in cells showing a low metastatic potential, interference with LASS2 may reduce the number of LASS2 molecules bound to ATP6L protein, rather than directly regulating the expression level of ATP6L. In turn, the increased number of unbound ATP6L molecules may play a role through other molecular mechanisms that promote apoptosis.

Increases in the H<sup>+</sup> concentration resulting in a decrease in pH is a type of external stimuli for cells, and induce a state of cellular stress (21). It has previously been reported that LASS2 is able to bind with ATP6L to inhibit H<sup>+</sup> from being pumped out of cells, resulting in a decline in the pH<sub>i</sub> value (10). ATP6L is a mediator of intracellular signaling cascades and can induce the collapse of the mitochondrial membrane potential, which can trigger a series of mitochondria-associated events, such as apoptosis (22). The results of the present study confirmed that si-LASS2 was able to significantly decrease Dox-induced apoptosis. Dox-induced apoptosis occurs initially via the c-Jun n-terminal kinase signaling system, which regulates the expression of nuclear transcription factors to affect cells. In particular, the P53 protein is phosphorylated, the expression of B-cell lymphoma-2 (Bcl-2)-associated X protein is increased and that of Bcl-2 is decreased, which promotes the apoptosis of cells (23). In the present study, LASS2 was shown to regulate the activity of ATPase and affect the H<sup>+</sup> concentration inside and outside of the cells, but it did not affect the expression levels of ATP6L. These results suggested that, in BC, LASS2 may bind to ATP6L to promote BC cell apoptosis and inhibit the growth of BC cells.

LASS2 genes have a Toll-IL-1-resistance domain-containing adaptor-inducing IFN-β-related adaptor molecule, LAG1 and ceroid-lipofuscinosis, neuronal 8 structural domain, which is necessary for the synthesis of ceramide, and is indispensable in the process of acetyl-CoA-dependent ceramide synthesis (24). Ceramide is an important second messenger molecule in cell signal transduction pathways, participating in the activation of various protein kinases and phosphatases to regulate cell growth, differentiation and apoptosis (25). Whether these

pathways play roles in the growth, invasion and metastasis of BC remain to be elucidated.

In conclusion, the present study confirmed that LASS2 was able to regulate V-ATPase activity and pH<sub>i</sub> through by directly interacting with the C subunit of V-ATPase to influence the apoptosis and proliferation of BC cells. Furthermore, upon further study, LASS2 may induce ceramide synthesis and increase cellular ceramide levels to stimulate cell apoptosis, which may provide important clues for understanding the role of LASS2 in BC cells. The results of the present study suggested that LASS2 expression may be correlated with the development and progression of human BC and may be a potential prognostic indicator for this cancer.

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