ARTICLE
Journal of Cellular Biochemistry 115:731–743 (2014)

Journal of Cellular Biochemistry

Silencing of LASS2/TMSG1 Enhances Invasion and Metastasis Capacity of Prostate Cancer Cell

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ABSTRACT

Homo sapiens longevity assurance homolog 2 of yeast LAG1 (LASS2), also known as tumor metastasis suppressor gene 1 (TMSG1), was firstly cloned by our laboratory in 1999. However, its antitumor molecular mechanisms are still unclear. LASS2/TMSG-1 could directly interact with the C subunit of Vacuolar H^+ ATPase (V-ATPase), which suggested that LASS2/TMSG1 might inhibit the invasion and metastasis through regulating the function of V-ATPase. In this study, we explored the effect of small hairpin RNA (shRNA) targeting LASS2/TMSG1 on the invasion and metastasis of human prostate carcinoma cell line PC-3M-2B4 with low metastatic potential and its functional interaction with V-ATPase. Silencing of LASS2/TMSG1 gene in PC-3M-2B4 cells increased V-ATPase activity, extracellular hydrogen ion concentration and in turn the activation of secreted MMP-2 and MMP-9, which coincided with enhancing cell proliferation, cell survival, and cell invasion in vitro, as well as acceleration of prostate cancer (PCA) growth and lymph node metastases in vivo. Thus we concluded that silencing of LASS2/TMSG1 enhances invasion and metastasis of PCA cell through increase of V-ATPase activity. These results establish LASS2/TMSG1 as a promising therapeutic target for advanced PCA. J. Cell. Biochem. 115: 731–743, 2014.

KEY WORDS: LASS2/TMSG1; PROSTATE CARCINOMA; PC-3M-2B4 CELLS; shRNA; VACUOLAR H^+ ATPase

Prostate cancer (PCA) is the principal malignant tumor threatening the health of senile male all over the world at present. Meanwhile, it is estimated that PCA accounts for about 33% (241,740) of new cancer cases and about 28,170 Americans will die from PCA every year, which is the second most common fatal cancer among men [Siegel et al., 2012]. Despite it is a slow growing carcinoma, and has a very high longer survival, the invasion and metastasis are still considered as the main causes of death for patients with PCA. Therefore, the study of the mechanism of PCA metastasis and the search for genes associated with PCA metastasis and therapeutic targets are in urgent demand.

Tumor metastasis suppressor gene 1 (TMSG1, GenBank accession number AF189062), Which is also recognized as LASS2 (*Homo sapiens* longevity assurance homolog 2 of yeast LAG1) or CerS2 (ceramide synthase 2), was firstly cloned as a novel tumor suppressor gene in our laboratory from a human PCA cell line PC-3M in 1999 and was designated as LASS2 in 2001 [Liu et al., 1999; Pan et al., 2001; Ma et al., 2002]. LASS2/TMSG1 is located on human chromosome 1, 1q21.3, and transcribed as two variants (no. NM_181746 and NM_022075) which both encode an identical protein of 380 amino acids with 45 kDa molecular weight (accession number of AAH10032) [Pei et al., 2004]. LASS2/TMSG1 protein has six protein kinase phosphorylation sites, a homeodomain (amino acids 71–128) and a TLC structural domain (amino acids 131–332), which forms five transmembrane domains [Xu et al., 2010]. Evaluation with monoclonal antibody has demonstrated that LASS2/TMSG1 protein is located in the endoplasmic reticulum of cells in the liver and kidney, and in the cytoplasm and membranes of tumor cells [Mizutani

Grant sponsor: National Sciences Foundation of China; Grant number: 30971142.

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Manuscript Received: 17 September 2013; Manuscript Accepted: 6 November 2013

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 15 November 2013

DOI 10.1002/jcb.24716 • © 2013 Wiley Periodicals, Inc.

et al., 2005; Laviad et al., 2008]. In addition to its fundamental function as a synthase of long-chain ceramide, the LASS2/TMSG1 protein plays a prominent role in suppression of tumor invasion and metastasis [Laviad et al., 2008]. It has been found that LASS2/TMSG1 is highly expressed in the low metastatic potential PCA cell line PC-3M-2B4 and shows low expression in the highly metastatic PCA cell line PC-3M-1E8, demonstrating a negative correlation with tumor metastatic potential. The roles played by LASS2/TMSG1 in suppression of tumor invasion and metastasis have been reported to be achieved through various mechanisms. LASS2/TMSG1 has been shown to directly bind to C subunit of the Vacuolar H^+ ATPase (V-ATPase) [Pan et al., 2001; Xu et al., 2010].

V-ATPase is an important pH regulatory complex in tumor cells and positively correlated to cancer invasion and metastasis, it is required to mediate signaling pathways, such as the Wnt pathway. V-ATPase uses the energy produced by ATP hydrolysis to pump protons into the extracellular environment or into the lumen of acidic vacuoles and thus play an important role in formation and maintenance of the extracellular acidic microenvironment [Sennoune et al., 2004]. These mechanisms were involved in activation of V-ATPase as well as consequent increase of cell movement and migration, the release of signal molecules attributing to degradation of extracellular matrix and the increase of V-ATPase-mediated sensitivity of cells to these molecules [Liotta and Kohn, 2001; Chen et al., 2004]. Recent studies show that LASS2/TMSG1 might increase intracellular H^+ of hepatocellular carcinoma via the interaction with V-ATPase, thereby induce cell apoptosis through mitochondrial pathway [Tang et al., 2010]. In addition, LASS2/TMSG1 also promotes cell apoptosis through the synthesis of ceramide, which in turn induces cell cycle arrest and affects the activity of telomerase as well as cell life span [Pettus et al., 2002; Ruvolo, 2003; Rossi et al., 2005; Laviad et al., 2008; Su et al., 2008].

RNA interference (RNAi) has emerged as a powerful tool to induce loss-of-function phenotypes by posttranscriptional silencing of gene expression [Fire et al., 1998]. In our previous study, we used small interfering RNAs (siRNAs) targeting LASS2/TMSG1 to explore the molecular mechanisms of LASS2/TMSG1 gene and obtained encouraging results [Xu et al., 2012]. Therefore, in this study, we used the DNA vector system to deliver four specially designed small hairpin RNAs (shRNAs) targeting LASS2/TMSG1 gene into human prostate carcinoma cell line PC-3M-2B4 with low metastatic potential to further evaluate the inhibitory effect of the LASS2/TMSG1 on growth, invasion and metastasis of PCA cell. Meanwhile, we elucidated the possible mechanisms relevant to LASS2/TMSG1′s antitumor effect on metastasis of PCA by investigating the activity of V-ATPase and the expression and activity of matrix metalloproteinase 2 (MMP-2), matrix metalloproteinase 9 (MMP-9) in the stable transfected cells.

MATERIALS AND METHODS

ETHICS STATEMENTS

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Peking University Health Science Center. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

CELLS AND CULTURE

Human PCA cell line PC-3M-2B4 cells (lowly metastatic variant of human prostate carcinoma cell line PC-3M; Tumorigenicity frequency in nude mice: 87.5%; Spontaneous metastasis frequency in nude mice: 0%) were established by Molecular Pathology Laboratory, Department of Pathology, Peking University Health Science Centre [Ma et al., 2002]. Cells were cultured in RPMI 1640 medium containing 10% quality fetal bovine serum (FBS, purchased from Hyclone, Logan, UT), 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in 5% CO_2. Experiments in vitro were done in this culture condition, unless indicated otherwise.

GENERATION OF PROSTATE CANCER CELL LINES STABLY EXPRESSING LASS2/TMSG1 SHORT HAIRPIN RNA

To inhibit the expression of LASS2/TMSG1, we designed four short hairpin RNAs (shRNA) according to the human LASS2/TMSG1 mRNA (Genebank accession no. NM181746.3). The LASS2/TMSG1 shRNA duplex with the following sense and antisense sequences were shown as Table I (sequencing 1 targeting LASS2/TMSG1 nucleotides 915–935, sequencing 2 targeting LASS2/TMSG1 nucleotides 1,123–1,143, sequencing 3 targeting LASS2/TMSG1 nucleotides 1,407–1,427, and sequencing 4 targeting LASS2/TMSG1 nucleotides 832–852). The four shRNA cassettes were subcloned into pGenesil vector (Genesil Biotechnology) and transfected into PC-3M-2B4 cells. As a negative control, the scrambled plasmid was designed to encode a hairpin RNA containing the same percentage of bp as shRNA plasmid, but in

TABLE I. Sequence of shRNA

shRNA	The two complementary oligonucleotides
shRNA-1-S	5′-CACC **GAAGAAAGTTTGGGAGGGATA** *TTCAAGACG* **TATCCCTCCCAAACTTTCTTC** TTTTTG-3′
shRNA-1-AS	5′-AGCTCAAAAAA **GAAGAAAGTTTGGGAGGGATA** *CGTCTTGAA* **TATCCCTCCCAAACTTTCTTC**-3′
shRNA-2-S	5′-TTTG **ACTCTAATCATGGCTCTGCAT** *TTCAAGACG* **ATGCAGAGCCATGATTAGAGT** TTTTT G-3′
shRNA-2-AS	5′-AGCTCAAAAAA **ACTCTAATCATGGCTCTGCAT** *CGTCTTGAA* **ATGCAGAGCCATGATTAGAGT**-3′
shRNA-3-S	5′-CCTCG **CCACAAGTTCATAACTGGAAA** *TTCAAGACG* **TTTCCAGTTATGAACTTGTGG** CTTTTTG-3′
shRNA-3-AS	5′-AGCTCAAAAAAGCCACAAGTTCATAACTGGAAA *CGTCTTGAA* **TTTCCAGTTATGAACTTGTGG** C-3′
shRNA-4-S	5′-CACCAAGCCAGCTGGAGATTCACATTT *TTCAAGACG* **AAATGTGAATCTCCAGCTGGC** TTTTTTTG-3′
shRNA-4-AS	5′-AGCTCAAAAAAAA **GCCAGCTGGAGATTCACATTT** *CGTCTTGAA* **AAATGTGAATCTCCAGCTGGC**TT-3′

Note: The 21-nucleotide sense or antisense strand is in bold, and stem loop sequences are in italics.

random sense sequence (GACTTCATAAGGCGCATGC), and did not target any known gene. Stable cell clones with reduced LASS2/TMSG1 expression were generated.

TRANSFECTION

Transfection of plasmid DNAs into PC-3M-2B4 cells was done using Lipofectamine™ (Invitrogen, Carlsbad, CA). Briefly, 1×10^5 cells were seeded per well into six-well plates (Corning, Inc., Corning, NY). The cells were transfected using 2 μg DNA mixed with 5 μl Lipofectamine™ in 1 ml medium without serum and antibiotics. The cells were incubated for 24 h, and then 1 ml medium containing 20% serum was added into each well. The cells were cultured and screened in the medium containing 10% serum and 3 mg/ml G418 (Invitrogen) for at least 2 weeks until the nontransfected PC-3M-2B4 cells cultured in the controlled wells were all killed. The stable transfectants, obtained by transfecting the LASS2/TMSG1 shRNA plasmid or scrambled plasmid into the PC-3M-2B4 cells, were designated as LASS2/TMSG1 shRNA or control shRNA, respectively.

WESTERN BLOT ANALYSIS

Cells (5×10^5) were planted in 25 cm^2 cell culture dishes and cultured in HCO_3^--buffered 1640 medium (pH 7.0) containing 5% FBS for 24 h. The supernatant was collected after 24 h culture, at the same time, total cellular protein was extracted. The protein concentration was determined using the BCA method (Pierce). Ten micrograms of protein from each sample was added to each lane, separated in 10% SDS–PAGE, and then transferred to NC membranes. The membranes were incubated overnight at 4°C in PBST and 5% dry milk containing polyclonal goat antihuman-LASS2/TMSG1 antibody (1:2,000, Santa Cruz), or polyclonal rabbit antihuman-MMP-2 antibody (1:1,000, CST), or polyclonal rabbit antihuman-MMP-9 antibody (1:500, CST), or monoclonal mouse antihuman-β-actin (1:2,500, Santa Cruz) as a control. The membranes were then washed and incubated for 2 h at room temperature in PBST and 5% dry milk containing a horseradish peroxidise-conjugated rabbit antigoat secondary antibody, or goat antirabbit, or goat antimouse (1:5,000, Santa Cruz), washed and incubated for 5 min with SuperSignal West Femoto Maximum Sensitivity Substrate (Pierce), and then exposed to X-ray films.

ACTIVITY OF V-ATPASE

For the preparation of microsomes, cultured cells were collected and homogenized in the medium containing 10 mmol/L HEPES (pH 7.4), 0.25 mol/L sucrose, 1 mmol/L DTT, 1 mmol/L PMSF, 1 mmol/L EDTA, 10 mmol/L KCl. After homogenization, the mixture was centrifuged at 700*g* for 2 min, and then 10,000*g* for 15 min. Supernatant was collected. The pellet was resuspended in the medium and homogenized again, and centrifuged same as above. Supernatant was collected together and centrifuged at 80,000*g* for an hour to precipitate the microsomal fraction. The pellet was resuspended in the medium (10 mmol/L HEPES (pH 7.4), 0.25 mol/L sucrose, 1 mmol/L DTT, 1 mmol/L PMSF), and stored at −80°C. All these manipulations were performed at 4°C. The protein concentration was determined according to the Bicinchoninic Acid assay (BCA) by the spectrophotometer. One hundred milligrams of microsome proteins was added to 700 ml reaction medium (containing 10 mmol/L HEPES–Tris pH 7.0, 0.2 mol/L sucrose, 50 mmol/L KCl, 1 mmol/L EDTA, 3 mmol/L ATP, 0.1 mmol/L ammonium molybdate, 5 mmol/L valinomycin, 5 mmol/L Nigericin, 5 mg/mL oligomycin, 1 mmol/L vanadate). The reaction was started with 5 mmol/L $MgSO_4$ at 37°C for 2 min. To evaluate V-ATPase activity, the release of phosphate was measured in spectrophotometer by colorimetric assay according to the manufacturer's instruction (Genmed Scientifics, Inc.). Absorbency (340 nm) of samples at the 0 min, the 5th min after beginning of the reaction was measured in reaction medium of malachite green to determine the V-ATPase activity. The experiment was repeated three times.

EXTRACELLULAR H^+ CONCENTRATION

The ability of proton secretion was determined by measuring pHe. pHe was measured using pH-sensitive dye BCECF (Sigma Chemical Co., St. Louis, MO). After the cells in the exponential phase were seeded in six-well plate for 12 h, the medium was removed after the cells attached to the plate and the cells were cultured in HCO_3^--buffered 1640 medium (pH 7.0) containing 5% FBS at 37°C in 5% CO_2 for 5 h. Then, the cells were washed twice using 0.9% NaCl, 120 μl serum-free HCO_3-buffered 1640 medium (1 mmol/L $NaHCO_3$ [pH 7.0]) was added into each well, and cells were incubated at 37°C in 5% CO_2. After the cells were cultured for 8, 12, or 24 h respectively, 100 μl supernatant per well was collected and 1 μmol/L BCECF was added into each sample. Absorbency (535 nm) of samples was measured by the Perkin-Elmer LS-50B. pHe was calibrated with the curve plotted by the fluorescence ratio F490/F440 of standard 1640 medium containing 1 μmol/L BCECF with a series of pH buffered by HCO_3-pHe value was converted to the extracellular proton concentration [Lu et al., 2005].

ACTIVITY OF MATRIX METALLOPROTEINASE-2 AND METALLOPROTEINASE-9 BY GELATIN ZYMOGRAPHY

The supernatant of each sample containing 20 μg protein was fractioned on 10% SDS–PAGE containing 0.1% A-type gelatin. After electrophoresis, gels were washed for 30 min in washing buffer (50 mmol/L Tris–HCl, 2.5% Triton X-100, 5 mmol/L $CaCl_2$ [pH 7.5]) and incubated in buffer (50 mmol/L Tris–HCl, 1% Triton X-100, 5 mmol/L $CaCl_2$ [pH 7.5]) for 20 h. The gel was observed on gel imaging analysis system.

MTT CELL PROLIFERATION ASSAY

We seeded cells in a 96-well plate at a density of 1,000 cells per well. At indicated time points, we added 10 μl of methylthiazoletetrazolium (MTT) solution (5 mg/ml in phosphate-buffered saline [PBS]). After 2 h of incubation, we added 100 μl of dimethyl sulfoxide (DMSO) to dissolve the crystals. We allowed the plate to stand for 10 min at room temperature and we recorded the absorbance at 540 nm.

SOFT AGAR ASSAY

PC-3M-2B4 cells transfected with LASS2/TMSG1 shRNA or control shRNA were mixed into 0.5 ml of 0.35% agar containing growth medium and layered over a base of 0.5% agar to prevent anchorage-dependent cell growth. Once this layer was solidified, it was overlaid with 1 ml of normal growth medium, which was replaced every 2 days for 14 days. A colony is defined as a cell aggregate larger than

100 μm. Pictures were taken and visible colonies were counted after 28 days.

APOPTOSIS ANALYSIS

Cells were digested by trypsin and washed twice by PBS. 300-mesh-nylon net was used to filter the cells. Binding buffer (200 μl) and 10 μl Annexin-V-FITC (Gene Research Center of Peking University) were used to make the resuspension of cells and incubated at 4°C for 30 min. Cells were dyed by 5 μl PI and 300 μl binding buffer before detected by the flow cytometer.

CELL CYCLE ANALYSIS

Cells were digested by trypsin and washed twice by PBS and then fixed by 75% alcohol in 4°C for 24 h. 300-mesh-nylon net was used to filter the cells and then 10 μl RNase was added, and incubated at 37°C for 30 min. Cells were dyed by PI before detected by the flow cytometer (Becton, Dickinson and Company, BD).

INVASION ASSAY

2×10^5 cells (400 μl) in exponential growth phase were plated on rehydrated Matrigel-coated culture inserts with 8 μm diameter pore size membranes (BD BioCoat™ Matrigel™ Invasion Chambers; BD Biosciences, Bedford, MA) in 24-transwell cell culture dishes. The bottom chamber contained 200 μl of NIH3T3-conditioned serum-free medium. After incubation for 12 h at 37°C in a humidified 5% CO_2 incubator, nonmigrated cells remaining in the insert were removed with a cotton swab. Migrated cells on the bottom of the filters were stained with hematoxylin and eosin (H&E) for 30 min. Cells on the filters were counted using a microscope. Triplicate filters were counted in each experiment.

TUMOR XENOGRAFTS

We injected 200 μl cell suspension of untransfected cells (1×10^6), negative control shRNA cells (1×10^6), and LASS2/TMSG1 shRNA cells (1×10^6) subcutaneously into the flank of athymic nude mice (6 mice/group) and measured the tumor dimensions by caliper once every week. The tumor volumes were calculated using the following formula: [length (mm) × width (mm) × width (mm) × 0.52]. At 8th week, tumors, lymph nodes, liver, and lungs were harvested from mice and H&E and immunohistochemistry stainings were evaluated. Animal Use Committee of the Peking University Health Science Center approved all protocols for treating animals.

IMMONOHISTOCHEMISTRY

Tumor and lymph node specimens were fixed in formalin overnight and embedded in paraffin. Series sections of 4 μm thick were prepared for immunohistological staining. Endogenous peroxidase was quenched by freshly prepared 3% H_2O_2 with 0.1% sodium azide and then placed in antigen retrieval solution (0.01 mol/L citrate buffer, pH 6.0) for 15 min in a microwave oven at 100°C and 600 W. After incubation in the casein block, goat polyclonal antihuman-LASS2/TMSG1 antibody (clone sc-65102, 1/200, Santa Cruz), or pan-cytokeratin (clone AE1/AE3, 1/50, Dako), or Ki-67 (clone GMD001, 1/100, Dako) was applied to the sections for an hour at room temperature, followed by incubation with second antibodies with horseradish peroxidase (1/200, Santa Cruz) for half an hour. The immune reaction was revealed with diaminobenzidine tetrachloride, and slides were counterstained with hematoxylin, dehydrated, and mounted. Consistent negative control was obtained by replacement of primary antibody with PBS.

STATISTICAL ANALYSIS

Two-tailed Fisher's exact test was used for analysis of positive ratio of tumor metastasis; two-tailed Student's *t*-test was used for other data. $P < 0.05$ was considered to be of statistical difference.

RESULTS

GENERATION OF PROSTATE CANCER CELL STABLY EXPRESSING LASS2/TMSG1 SHRNA

PC-3M-2B4 cells, the prostate carcinoma cells with low metastatic potential, were transfected with the constructed plasmid encoding shRNA targeting LASS2/TMSG1 and with negative controlled plasmid containing scrambled random sequence, respectively. The stable transfectants with LASS2/TMSG1 shRNA and negative control shRNA were established by G418 screening. A single clone expressing LASS2/TMSG1 shRNA derived from PC-3M-2B4 cells showed nearly 100% inhibition of LASS2/TMSG1 protein by Western blotting. The expression of LASS2/TMSG1 did not change between the cells transfected with negative control shRNA and the untransfected cancer cells (Fig. 1).

SHRNA AGAINST LASS2/TMSG1 INCREASE THE ACTIVITY OF V-ATPASE IN PC-3M-2B4 CELLS

The activity of V-ATPase was detected by GEMEND's V-ATPase activity assay kit. The activity of V-ATPase = OD of sample/(concentration × 31.1) × 10^3 U/mg. The results showed that the V-ATPase activity in LASS2/TMSG1 shRNA cells increased with statistical significance compared with untransfected cells or control shRNA cells ($P < 0.05$, n = 3), as shown in Table II and Figure 2A.

LASS2/TMSG1 SHRNA INCREASE EXTRACELLULAR H^+ CONCENTRATION IN PC-3M-2B4 CELLS

Extracellular H^+ concentration was detected by pH-sensitive fluorescence probe bis-carboxy-ethyl-carboxy-fluorescein (BCECF), as shown in Figure 2B. The proton secretion of the LASS2/TMSG1 shRNA cells was notably increased at 12 and 24 h compared with that of control shRNA cells or untransfected cells ($P < 0.01$, n = 3).

LASS2/TMSG1 SHRNA INCREASES THE ACTIVITY OF MMP-2 AND MMP-9 IN THE SUPERNATANT OF THE CULTURE CELLS

We detected the expression, secretion level, and activity of MMP-2 and MMP-9 of the three group cells, which are closely related to cancer metastasis according to the previous reports [Lu et al., 2005]. Western blot showed that there was no significant difference in the expression and secretion of MMP-2 and MMP-9 protein among the LASS2/TMSG1 shRNA cells, control shRNA cells, or untransfected cells (Fig. 3A) in total protein and cell supernatant of the PC-3M-2B4 cells, indicating that the downregulation of LASS2/TMSG1 expression did not affect the expression and secretion of MMP-2, MMP-9 protein. (*Note*: the secretion of MMP-9 had not been detected possibly because of very low concentration.)

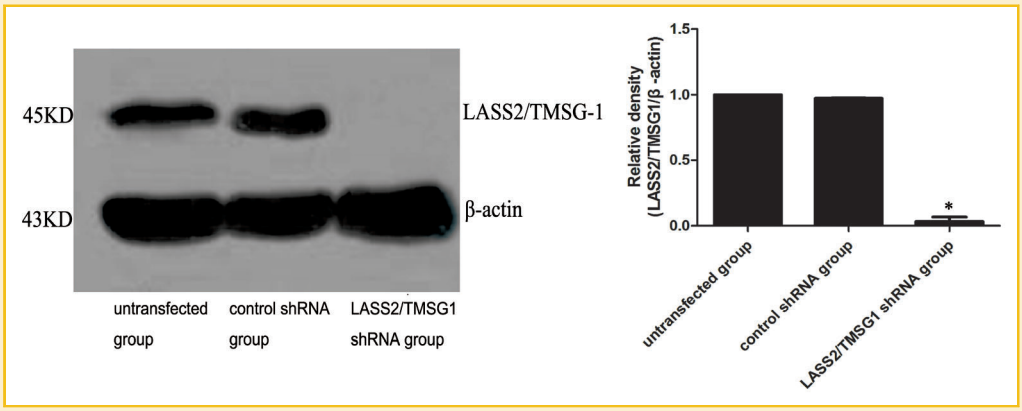

Fig. 1. Western blot revealed LASS2/TMSG1 protein expression was completely inhibited in the LASS2/TMSG1 shRNA group compared with that in the control shRNA group ($^{*}P < 0.05$).

Furthermore, the supernatant of cultured cells was collected and the gelatinase activity was assayed with gelatin zymography. The activity of MMP-2 and MMP-9 was apparently increased in the supernatant of the LASS2/TMSG1 shRNA cells compared with the control shRNA cells or untransfected cells (Fig. 3B–D), which indicated that the downregulation of LASS2/TMSG1 expression increased the activity of MMP-2 and MMP-9 protein.

LASS2/TMSG1 SHRNA-INDUCED CELL GROWTH ACCELERATION IN PC-3M-2B4 CELLS

The MTT assay demonstrated that LASS2/TMSG1 shRNA resulted in dramatic growth acceleration of PC-3M-2B4 cells, as compared with control shRNA or untransfected cells (Fig. 4A). This effect was more pronounced at 4, 5, and 6-day posttreatment.

SHRNA TARGETING LASS2/TMSG1 PROMOTED THE COLONY-FORMATION CAPACITY OF PC-3M-2B4 CELLS

Using soft agar assay, the LASS2/TMSG1 shRNA cells produced 1.5-fold more colonies relative to the control shRNA cells or untransfected PC-3M-2B4 cells (Fig. 4B). The colony numbers of control shRNA cells, untransfected cells and LASS2/TMSG1 shRNA cells in soft agar were 37.33 ± 4.76, 36.67 ± 3.51, and 56 ± 4.58, respectively, with statistically significant difference ($P < 0.05$, $n = 3$; Fig. 4C). Therefore, shRNA targeting LASS2/TMSG1 significantly increased the colony-formation capacity of PC-3M-2B4 cell.

LASS2/TMSG1 SHRNA DECREASED THE APOPTOSIS RATE OF PC-3M-2B4 CELLS

The effect of apoptosis rate by LASS2/TMSG1 shRNA on PC-3M-2B4 cells was further evaluated by flow cytometry analysis. LASS2/TMSG1 shRNA decreased the apoptosis rate from 18% and 19%, as seen in untransfected cells and control shRNA cells, to 12% as seen in the LASS2/TMSG1 shRNA treated PC-3M-2B4 cells, respectively ($P < 0.05$, $n = 3$; Fig. 5).

LASS2/TMSG1 SHRNA HAD LESS EFFECTS ON CELL CYCLE DISTRIBUTION

Flow cytometry analysis showed that there was no significant difference of cell cycle distribution of the LASS2/TMSG1 shRNA cells, control shRNA cells, or untransfected cells (Fig. 6), indicating that the downregulation of LASS2/TMSG1 expression did not affect the cell cycle distribution of PC-3M-2B4 cells.

SHRNA TARGETING LASS2/TMSG1 PROMOTED THE INVASION OF PC-3M-2B4 CELLS

Using Boyden chamber invasion assay, the LASS2/TMSG1 shRNA cells displayed dramatically increasing invasion ability compared with the control shRNA cells and untransfected cells (Fig. 7A). The cell numbers of invasion in the control shRNA cells, untransfected cells, and LASS2/TMSG1 shRNA cells were: 21 ± 1.0, 21.67 ± 2.52, and 52.33 ± 3.21, respectively. The difference was statistically significant ($P < 0.05$, $n = 3$; Fig. 7B).

TABLE II. V-ATPase Activity of Three Groups

	V-ATPase activity			
Cell lines	First	Second	Third	$\bar{X} \pm SD$
Untransfected group	1.578	1.271	1.369	1.406 ± 0.157
Control shRNA group	1.271	1.702	1.093	1.355 ± 0.313
LASS2/TMSG1 shRNA group[a]	2.369	2.963	3.002	2.778 ± 0.355

[a]The V-ATPase activity in LASS2/TMSG1 shRNA treated 2B4 cells increased significantly compared with that in untransfected cells and control shRNA cells ($P < 0.05$, $n = 3$).

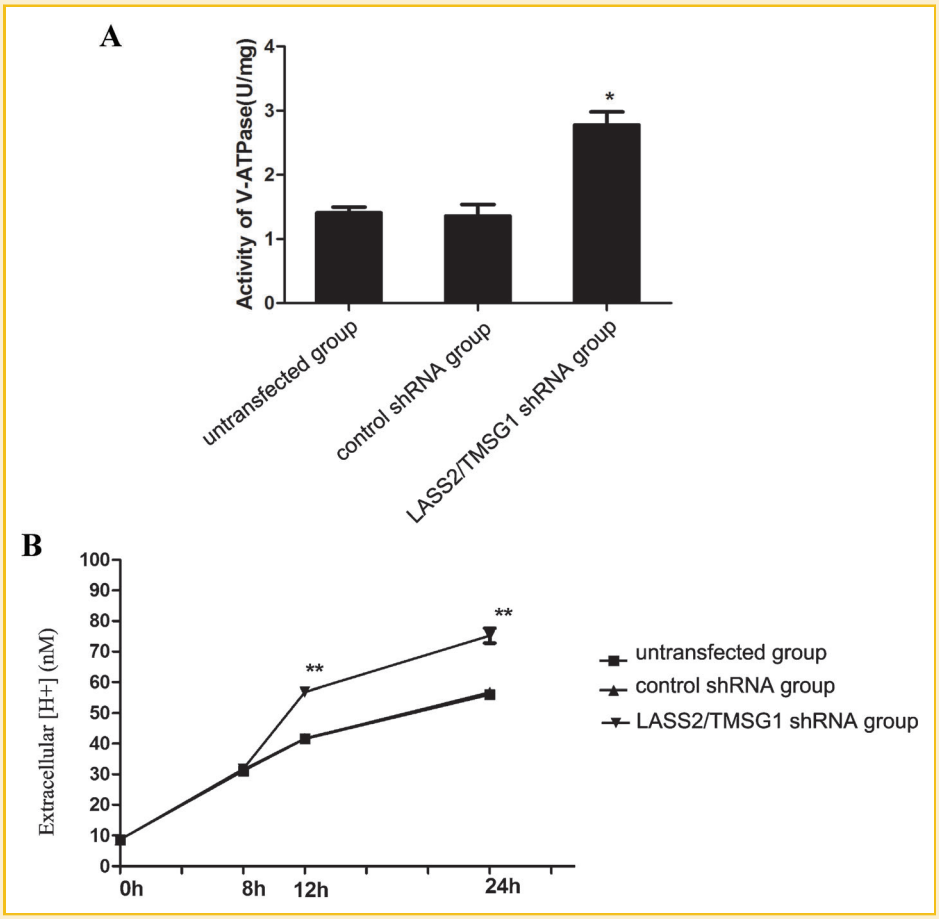

Fig. 2. The activity of V-ATPase and the extracellular [H^+] concentration in three groups of PC-3M-2B4 cells. A: The V-ATPase activity in LASS2/TMSG1 shRNA cells increased significantly compared with that in untransfected cells and control shRNA cells ($^*P < 0.05$, n = 3). B: Proton secretion at 12 and 24 h was notably increased in the LASS2/TMSG1 shRNA cells compared with that in untransfected cells and control shRNA cells ($^{**}P < 0.01$, n = 3).

LASS2/TMSG1 SHRNA-INDUCED ACCELERATION OF GROWTH, PROLIFERATION, INVASION, AND METASTASIS IN XENOGRAFTS

The LASS2/TMSG1 shRNA cells, control shRNA cells, or untransfected cells were subcutaneously injected into nude mice, respectively. The size of tumors was blindly measured once a week with calipers. After 8 weeks, the xenografts, lymph nodes, liver, and lungs were removed from mice and H&E and immunohistochemistry stainings were evaluated. By measuring, we found that the average size and weight of xenografts in LASS2/TMSG1 shRNA group were dramatically bigger and heavier than that of the control shRNA group or untransfected group ($^{**}P < 0.01$; Fig. 8; Table III). No difference was observed between the control shRNA group and untransfected group. By immunohistochemistry staining, we observed the xenografts lacked of LASS2/TMSG1 expression in the LASS2/TMSG1 shRNA group, compared with the untransfected group and the control shRNA group (Fig. 9A). Furthermore, immunohistochemistry of Ki-67 was performed in the xenografts and the percentages of Ki-67 were calculated. Approximately 1,000 cells were counted in each immunohistochemical section. Results are presented as means of five counts. There was no significant difference of Ki-67 index between the control group and untransfected group. However, the Ki-67 index of the LASS2/TMSG1 shRNA cells was strikingly higher than the control cells or untransfected cells (Fig. 9B). The Ki-67 index of xenografts in LASS2/TMSG1 shRNA group (0.83 ± 0.05) were notably higher than the control shRNA group (0.1 ± 0.03) and untransfected group (0.13 ± 0.02; $P < 0.05$).

Furthermore, there was higher lymph node metastasis in the LASS2/TMSG1 shRNA group (5/6) compared with the control shRNA (1/6) and the untransfected group (0/6; Fig. 10, Table IV). However, intrahepatic metastasis or pulmonary metastasis was not observed in the three groups. Furthermore, in the LASS2/TMSG1 shRNA group, we can more frequently observe the phenomena of intravenous tumor thrombus, atypical mitotic figures, and tumor invasion into muscle and nerve (Fig. 10, Table V), which indicated that LASS2/TMSG1 shRNA induced acceleration of growth, proliferation, invasion, and metastasis in xenografts.

DISCUSSION

LASS2/TMSG1 gene was firstly cloned as a novel TMSG by our laboratory from nonmetastatic and metastatic cancer cell variants of human prostate carcinoma by mRNA differential display in 1999

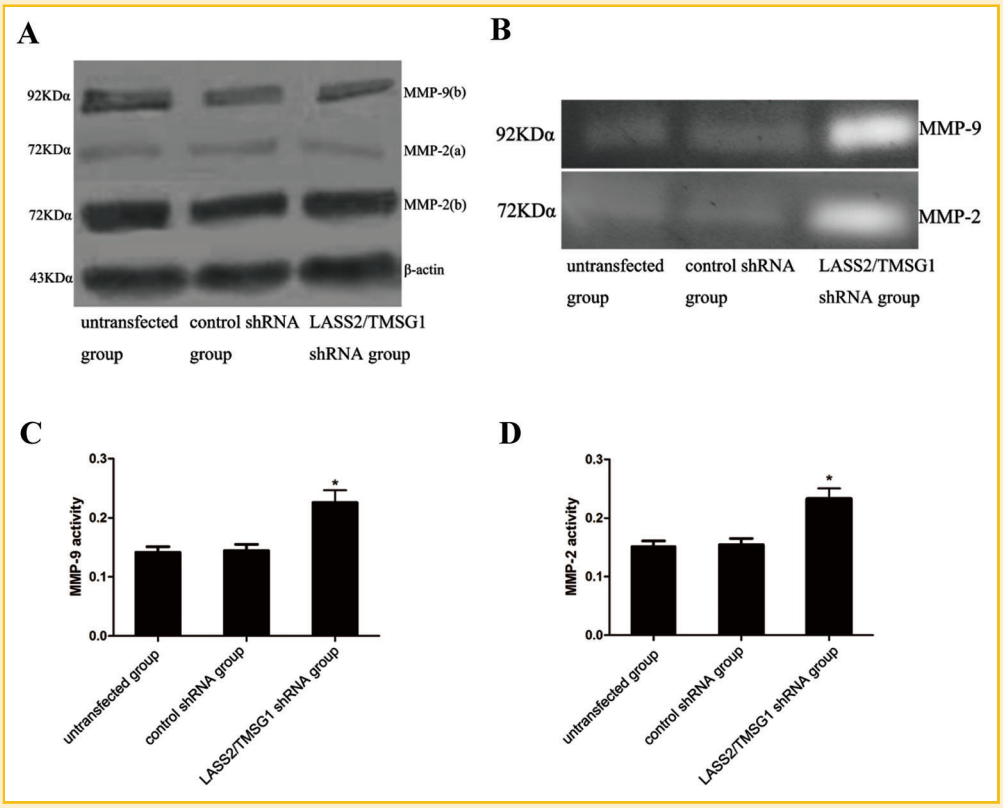

Fig. 3. The expression, secretion, and gelatinase activity of MMP-2 and MMP-9 in PC-3M-2B4 cells. A: MMP-2 and MMP-9 protein in the PC-3M-2B4 cells (b) and supernatant (a) was analyzed by Western blot, but there was no significant difference in the expression and secretion of MMP-2 and MMP-9 protein among the LASS2/TMSG1 shRNA cells, control shRNA cells or untransfected cells. (*Note*: the secretion of MMP-9 had not been detected possibly because of very low concentration.) B–D: the activity of MMP-2 and MMP-9 were examined by gelatin zymography. The activity of MMP-2 and MMP-9 in LASS2/TMSG1 shRNA group were obviously higher than that in the control shRNA or untransfected group ($^{*}P < 0.05$).

[Ma et al., 2002], with GenBank accession number AF189062 [Pan et al., 2001]. Using sense and antisense cDNA transfection, LASS2/TMSG1 was found to significantly suppress the invasion ability of human PCA cell line PC-3M, human pulmonary giant cell carcinoma cell line PG, and human breast cancer cell line MDA-MB-231 [Mizutani et al., 2005]. Shanghai research group had identified interaction between LASS2/TMSG1 and seven membrane-associated receptors or transporters by yeast two-hybrid screening and glutathione-*S*-transferase (GST) pull-down assays. Among them, four were confirmed to physically interact with LASS2/TMSG1 in the GST pull-down assay as described below: the high-affinity asialoglycoprotein receptors type 1 (AGPRH1) and type 2 (AGPRH2), the organic cation transporter-1 (OCT1) and the proteolipid subunit of V-ATPase, which suggested LASS2/TMSG1 was a membrane-association protein [Pei et al., 2004]. It was notable that LASS2/TMSG1 directly interacted with subunit C of V-ATPase.

TABLE III. The Volume and Weight of Xenografts

Group	Volume (cm^3)	Weight (g)
Untransfected group	0.3677 ± 0.0731	0.4817 ± 0.1132
Control shRNA group	0.3246 ± 0.0929	0.6267 ± 0.1356
LASS2/TMSG1 shRNA group	1.5739 ± 0.2675	1.3517 ± 0.1826

However, as a novel TMSG, the precise molecular mechanisms of LASS2/TMSG1 on cancer metastasis through V-ATPase are unclear. Therefore, to further investigate the molecular mechanisms of LASS2/TMSG1′s antitumor effect on metastasis of PCA, RNAi was adopted to silence the gene expression of LASS2/TMSG1 in human PCA cell line PC-3M-2B4 (low metastatic potential, high expression of LASS2/TMSG1).

To improve the stability of transfection and interference, we used the plasmid encoding four shRNA duplex targeting LASS2/TMSG1 in this experiment. In addition, tumorigenicity in nude mice experiments and agarose colony formation assay can not be achieved in transiently transfected cells, we can use stably transfected cells by LASS2/TMSG1 shRNA to detect biological function of LASS2/TMSG1 in vitro and in vivo to better reflect LASS2/TMSG1′s antitumor effect. In our experiments, we successfully constructed the LASS2/TMSG1 shRNA plasmid, and the inhibitory effect of LASS2/TMSG1 is up to 100% at the protein level. Then we detected the biological behavior of LASS2/TMSG1 shRNA stably transfected PC-3M-2B4 cells. Using Boyden chamber invasion assay, the LASS2/TMSG1 shRNA cells displayed dramatically increasing invasion ability when compared

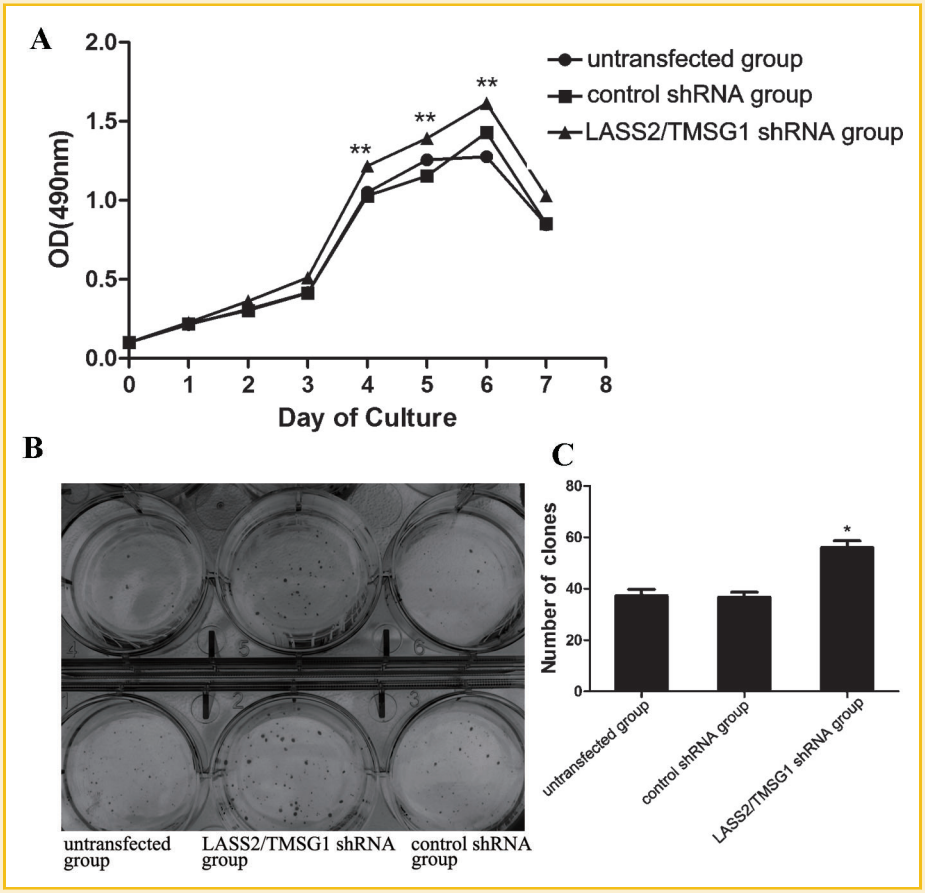

Fig. 4. MTT assay and soft agar assay shows the proliferation ability of PC-3M-2B4 cells. A: Compared with that in control shRNA cells and untransfected cells, LASS2/TMSG-1 shRNA caused a dramatic growth acceleration in PC-3M-2B4 cells. B: Soft agar assay shows that LASS2/TMSG1 shRNA cells had a clear increase of the colony-formation capacity. C: The colony numbers of LASS2/TMSG1 shRNA cells was more than that of control shRNA cells and untransfected cells, the difference was statistically significant ($^{*}P < 0.05$).

with the control shRNA cells and untransfected PC-3M-2B4 cells. The LASS2/TMSG1 shRNA cells, control shRNA cells, or untransfected PC-3M-2B4 cells were s.c. injected into nude mice, respectively. After 8 weeks, we can observe the phenomena of intravenous tumor thrombus, pathological mitosis, and tumor invasion for muscle, nerve in the si-PC-3M-2B4 group. The impressing effect of LASS2/TMSG1 shRNA is its striking increase of the metastatic potential of PC-3M-2B4 cells. In control, one of six mice had the lymph node metastasis, whereas in LASS2/TMSG1 shRNA-treated group, five of six had lymph node metastasis. This can prove that LASS2/TMSG1 is a TMSG from the opposite direction. The conclusion was in accordance with other laboratory. Shanghai research group found that LASS2/TMSG1 could inhibit the metastasis of human hepatocellular carcinoma HCCLM3 cells using sense cDNA transfection [Liu et al., 2004; Novina and Sharp, 2004; Tan et al., 2007]. Besides, they also discovered that small interfering RNA targeting TMSG1/LASS2 gene could enhance invasion capacity of human hepatocellular cell line MHCC97-L [Tang et al., 2009]. Furthermore, we used stably transfected cells to detect other biological function of LASS2/TMSG1 shRNA on PC-3M-2B4 cells in vitro and in vivo as follows. (1) The MTT assay demonstrated that LASS2/TMSG1 shRNA resulted in dramatic growth acceleration of PC-3M-2B4 cells. (2) Using soft agar assay, the LASS2/TMSG1 shRNA cells produced 1.5-fold more colonies relative to the control shRNA cells or untransfected PC-3M-2B4 cells. (3) LASS2/TMSG1 shRNA decreased the apoptosis rate of PC-3M-2B4 cells by flow cytometry analysis. However, LASS2/TMSG1 shRNA had less effects on cell cycle distribution. (4) LASS2/TMSG1 shRNA induced acceleration of growth and proliferation in xenografts. The above results reversely indicated LASS2/TMSG1 was a novel TMSG in human PCA. It can not only inhibit the invasion and metastasis capacity of tumor, but also play a role in the inhibition of tumor's growth and proliferation.

Furthermore, in order to investigate the tumor metastasis suppression mechanism of LASS2/TMSG1, we studied the interaction among LASS2/TMSG1, V-ATPase, and MMP. Our results showed as follows: (1) LASS2/TMSG1 shRNA could enhance the activity of V-ATPase by GEMEND's V-ATPase activity assay kit. (2) LASS2/TMSG1 shRNA increases extracellular H^{+} concentration using pH-sensitive BCECF. (3) LASS2/TMSG1 shRNA increases the activity of MMP-2 and MMP-9 in the supernatant of the cultured cells by gelatin zymography. The findings were confirmed by Shanghai research group. They found that LASS2/TMSG1 siRNA could increase the

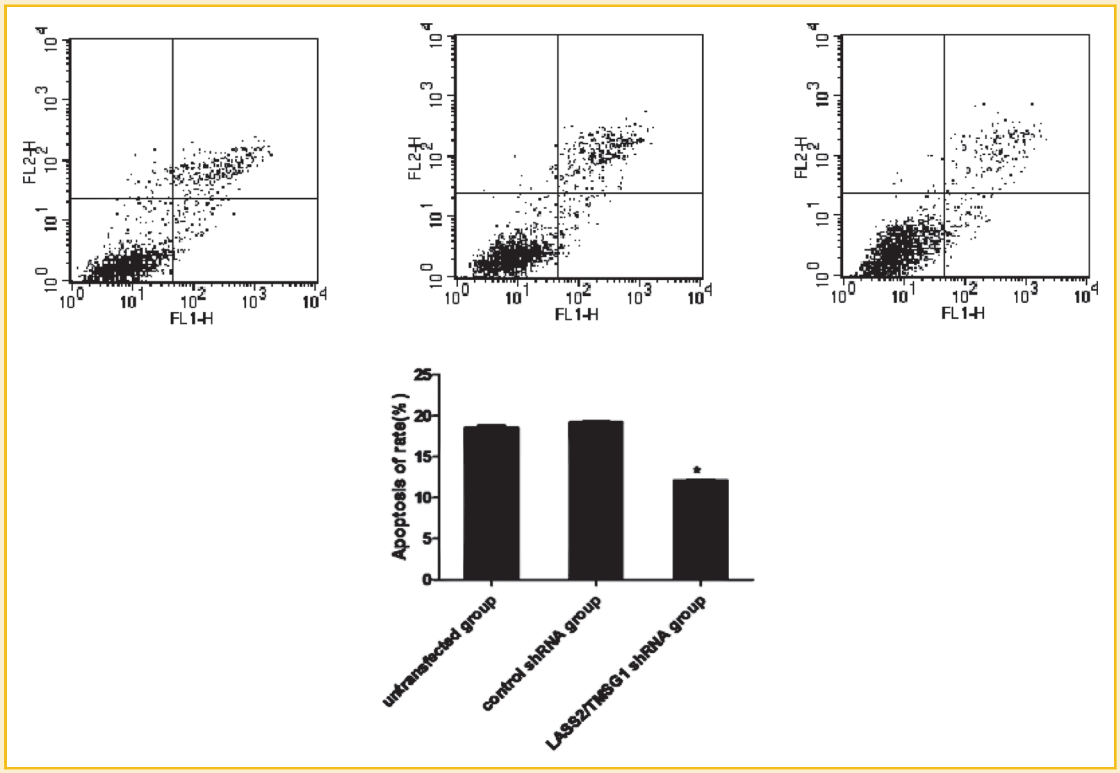

Fig. 5. Flow Cytometry showed LASS2/TMSG1 shRNA significantly decreased the apoptosis rate relative to the control shRNA cells or untransfected PC-3M-2B4 cells ($^{*}P < 0.05$, $n = 3$).

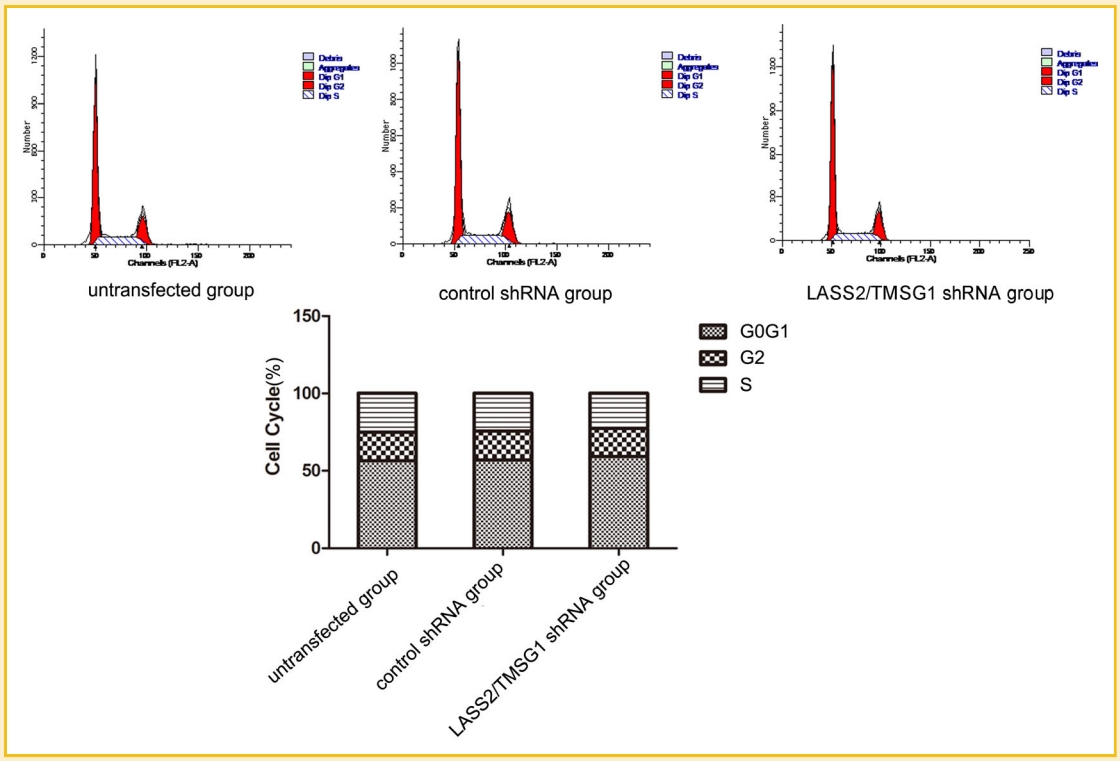

Fig. 6. Flow cytometry showed silencing of LASS2/TMSG-1 gene had no effect on the cell cycle distribution of PC-3M-2B4 cells.

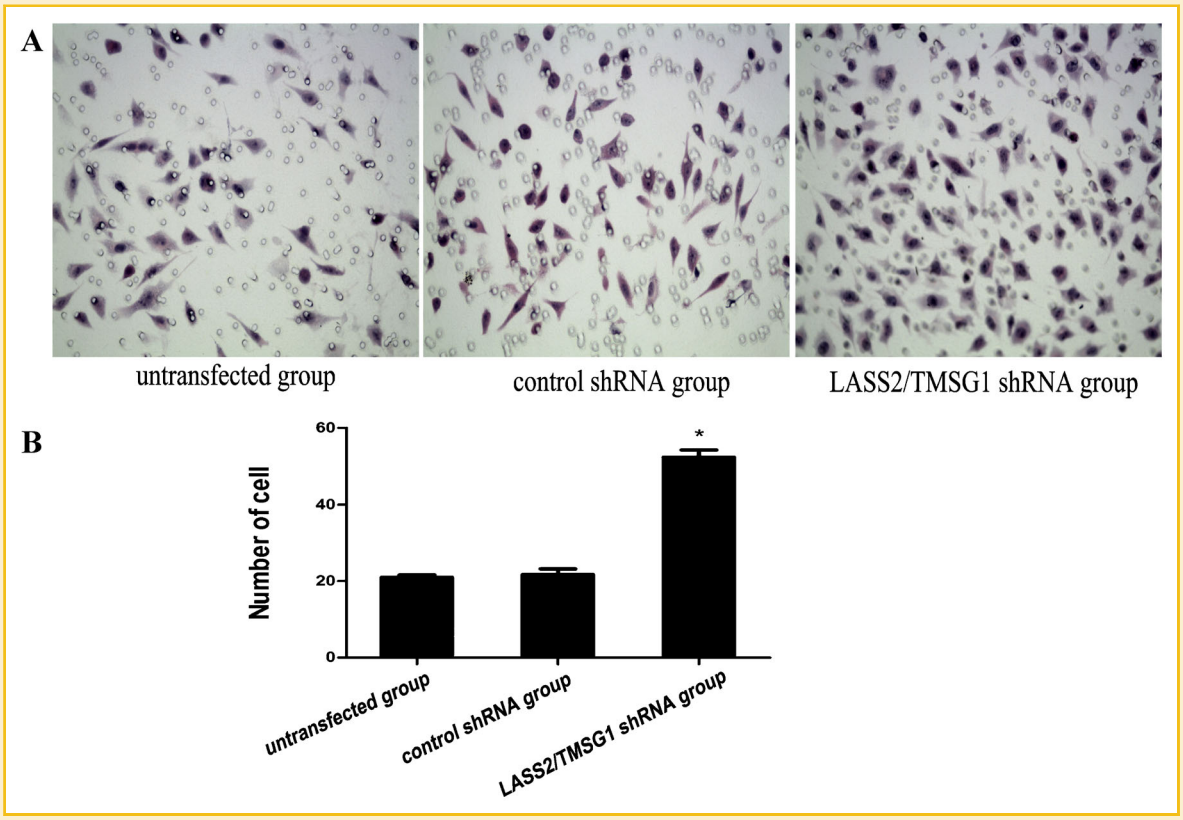

Fig. 7. Using Boyden chamber invasion assay, the LASS2/TMSG1 shRNA cells displayed dramatically increasing invasion ability compared with the control shRNA cells and untransfected cells ($^{*}P < 0.05$, n = 3), and there was no difference between control shRNA cells and untransfected cells.

proton transmembrane secretion in MHCC97-L cells by measuring extracellular pH with pH-sensitive BCECF [Tang et al., 2009] and LASS2 could enhance chemosensitivity of breast cancer by counteracting acidic tumor microenvironment through inhibiting activity of V-ATPase proton pump [Fan et al., 2012]. These results suggested that LASS2/TMSG1 could inhibit the activity of vacuolar ATPase, suppress the proton transmembrane secretion and thus decrease extracellular H^{+} concentration in cancer cells. The promoting effect

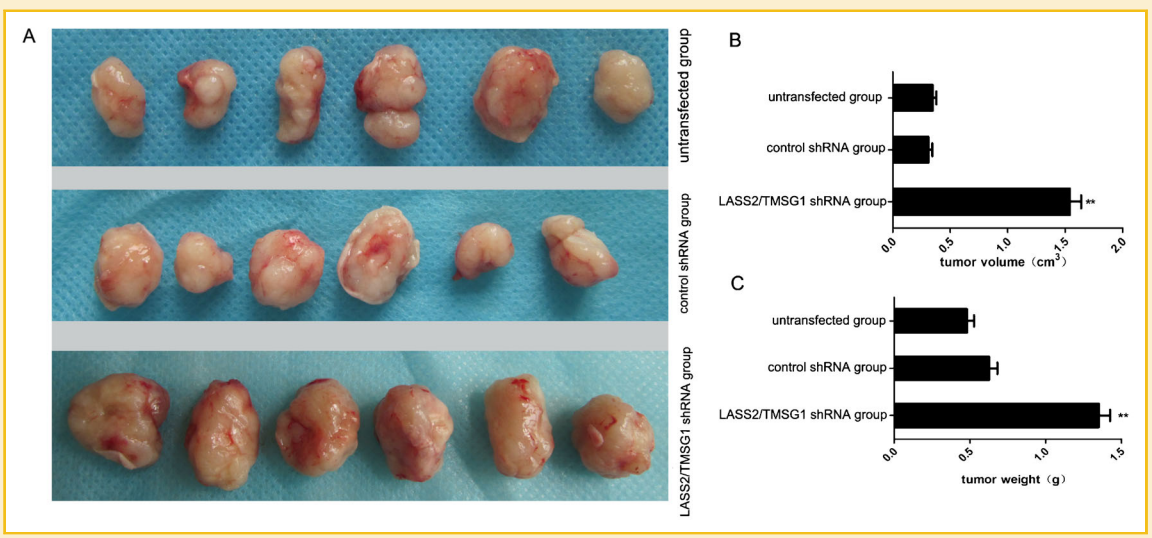

Fig. 8. The average size and weight of xenografts in LASS2/TMSG1 shRNA group were dramatically bigger and heavier than that of the control shRNA group or untransfected group ($^{**}P < 0.01$). No difference was observed between the control shRNA group and untransfected group.

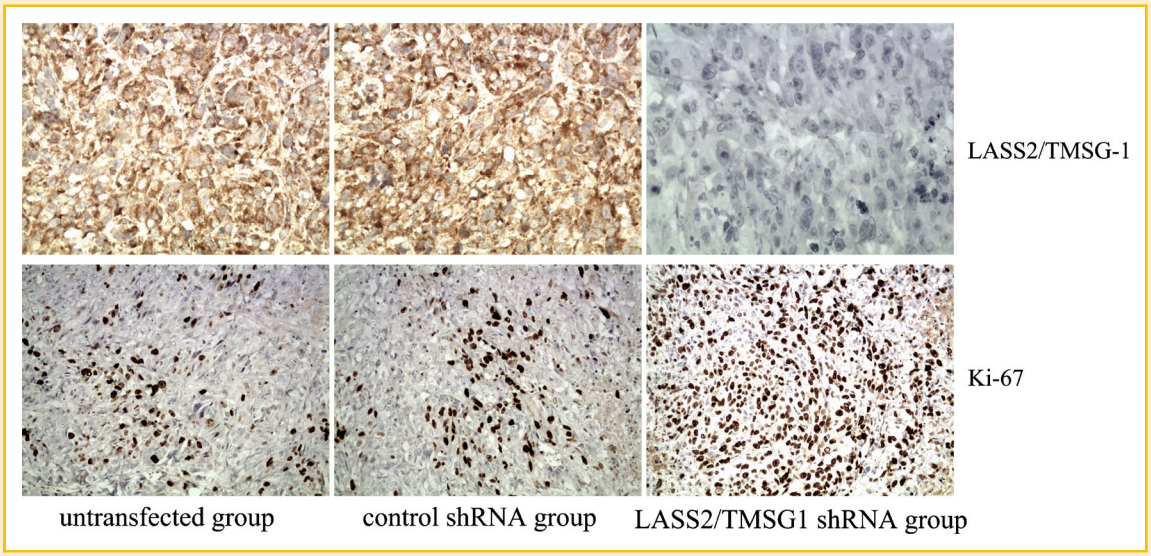

Fig. 9. Immunohistochemistry staining shows the xenografts lacked of LASS2/TMSG-1 expression in the LASS2/TMSG1 shRNA group, compared with that in the untransfected group and the control shRNA group; the Ki-67 index of xenografts in LASS2/TMSG1 shRNA group (0.83 ± 0.05) were notably higher than the control shRNA group (0.1 ± 0.03) and untransfected group (0.13 ± 0.02).

of V-ATPase on cancer invasion and metastasis mainly relies on its maintaining acidic pH of extracellular microenvironment and very acidic luminal pH [Lu et al., 2005], which is related to the activation, secretion, and cellular distribution of many proteases involved in the digestion of ECM. The pH-sensitive proteases include cathepsin [Montcourrier et al., 1994; Rozhin et al., 1994; Martinez-Zaguilan et al., 1996] and MMPs [Fasciglione et al., 2000; Johnson et al., 2000]. In this experiment, we discovered that shRNA against LASS2/TMSG1 could increase the activity of MMP-2, MMP-9 in the supernatant of cells, but had no effect on the expression and secretion of MMP-2, MMP-9 protein.

Moreover, when ECM is digested during invasion, some trapped factors that can regulate cellular proliferation, apoptosis, and adhesion will be released from the degraded ECM [Tang et al., 2010]. V-ATPase can also regulate the sensitivity of cells to these molecular signals by efficiently recycling of membrane receptor

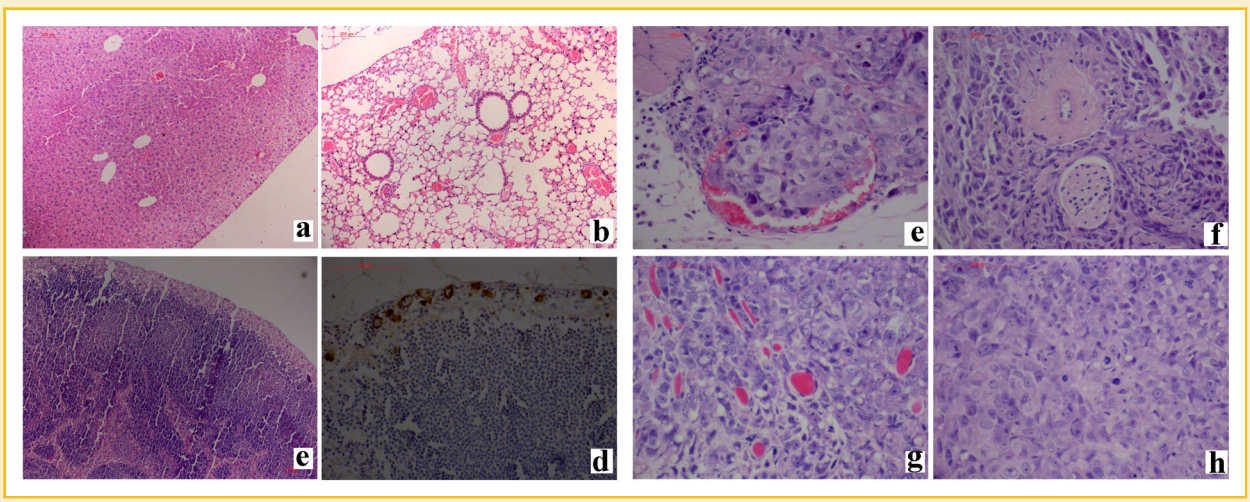

Fig. 10. Intrahepatic metastasis or pulmonary metastasis was not observed in the three groups (A: liver, B: lung). However, there was higher lymph node metastasis in the LASS2/TMSG1 shRNA group (5/6) compared with the control shRNA (1/6) and the untransfected group (0/6). C: Hematoxylin and eosin staining showed lymph node metastasis in the LASS2/TMSG1 shRNA group. D: Immunostaining for CK (AE1/AE3) showed positive expression of metastatic carcinoma cells in lymph node of the LASS2/TMSG1 shRNA group). Furthermore, in the LASS2/TMSG1 shRNA group, we can more frequently observe the phenomena of intravenous tumor thrombus (E), tumor invasion into nerve (F), muscle (G), and atypical mitotic figures (H).

TABLE IV. The Comparison of Metastatic Ratio Among the Untransfected Group, Control shRNA Group, and the LASS2/TMSG1 shRNA Group

		Control shRNA group		LASS2/TMSG1 shRNA group	
Metastasis	Untransfected group	Ratio	*P*	Ratio	*P*
Lymphatic	0/6, 0%	1/6, 16.7%	>0.05	5/6, 83.3%	<0.05
Intrahepatic	0/6, 0%	0/6, 0%	>0.05	0/6, 0%	>0.05
Pulmonary	0/6, 0%	0/6, 0%	>0.05	0/6, 0%	>0.05

Note: *P* indicates possibility compared with untransfected group using two-tailed Fisher's exact test.

TABLE V. The Growth and Invasion of Xenografts Among the Untransfected Group, Control shRNA Group, and the LASS2/TMSG1 shRNA Group

		Control shRNA group		LASS2/TMSG1 shRNA group	
Pathology indices	Untransfected group, ratio	Ratio	*P*	Ratio	*P*
Intravenous tumor thrombus	0/6, 0%	0/6, 0%	>0.05	5/6, 83.3%	<0.05
Tumor invasion for nerve	0/6, 0%	1/6, 16.7%	>0.05	4/6, 66.7%	<0.05
Tumor invasion for muscle	1/6, 16.7%	1/6, 16.7%	>0.05	3/6, 50%	>0.05
Mitotic figures (>10/10 HPF)	1/6, 16.7%	1/6, 16.7%	>0.05	6/6, 100%	<0.01

Note: *P* indicates possibility compared with untransfected group using two-tailed Fisher's exact test.

to the plasma membrane for reutilization. Furthermore, the C subunit of V-ATPase, ATP6L itself has binding sites for papillomavirus E5 oncoprotein, platelet-derived growth factor and integrin, which can also regulate the proliferation and adhesion [Lu et al., 2005]. In 2010, Tang et al. found that LASS2/TMSG-1 gene might increase intracellular H^+ of HCC cells via the interaction with V-ATPase, thereby inducing cell apoptosis through mitochondrial pathway. Furthermore, in 2012, Yu et al. found that LASS2/TMSG1 could regulate V-ATPase activity and intracellular pH through the direct interaction of its Homeodomain and the C subunit of V-ATPase in human PCA cell lines PC-3M-1E8 and PC-3M-2B4 cells. Their interaction could play important roles in the apoptosis of tumor cells. It was consistent with our results. In view of our experimental results, we speculated LASS2/TMSG1 shRNA may decrease intracellular H^+ of PC-3M-2B4 cells via the direct interaction with V-ATPase and thereby inhibit cell apoptosis and accelerate cell proliferation.

In conclusion, silencing of LASS2/TMSG1 gene can promote growth, proliferation, invasion, and metastasis of human PCA cell in vitro and in vivo through the increase of the V-ATPase activity and extracellular hydrogen ion concentration, and in turn the activation of secreted MMP-2, MMP-9, and degradation of ECM, indicating that LASS2/TMSG1 is a novel TMSG. Moreover, silencing of LASS2/TMSG1 may promote proliferation and inhibit apoptosis of human PCA cell in vitro and in vivo through the direct interaction with V-ATPase and thus the increase of extracellular H^+.

ACKNOWLEDGMENTS

The article was supported by the National Sciences Foundation of China (30971142). Thanks to Beiying Liu for data analysis.

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