

LASS2/TMSG1 inhibits growth and invasion of breast cancer cell in vitro through regulation of vacuolar ATPase activity

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Abstract Homo sapiens longevity assurance homologue 2 of yeast LAG1 (LASS2)/tumor metastasis suppressor gene 1 (TMSG1) was a novel tumor metastasis-related gene identified using messenger RNA differential display from non-metastatic human prostate cancer cell variants. The mechanism of LASS2/TMSG1 inhibiting tumor invasion metastasis in breast cancer cells had not been well investigated. In the present study, a full length of 1.2 kb LASS2/TMSG1 complementary DNA (cDNA) coding for a protein of 380 amino acids was cloned. PcDNA3 eukaryotic expression plasmids of LASS2/TMSG1 were constructed and transfected into human breast cancer cell line MCF-7 by lipofectin transfection method. And, the biological effects were observed comparing with control groups. As the result, LASS2/TMSG1 inhibited cell growth in vitro by increasing apoptosis and changing cell cycle distribution. Furthermore, the vacuolar ATPase (V-ATPase) activity and

extracellular hydrogen ion concentration were significantly decreased and the activity of secreted matrix metalloproteinase-2 (MMP-2) was downregulated in MCF-7 cells overexpressing LASS2/TMSG1 compared with the controls. Therefore, LASS2/TMSG1 may inhibit growth and invasion of breast cancer cell in vitro through decreasing V-ATPase activity and extracellular hydrogen ion concentration and inactivating secreted MMP-2. The findings provided the evidence that the LASS2/TMSG1 gene had tumor growth and invasion suppressor function in human breast cancer cell and may provide a promising target for cancer metastasis diagnosis and therapy.

Keywords LASS2/TMSG1 · Breast cancer · Vacuolar ATPase · MMP-2

Introduction

Breast cancer is the most prevalent cancer among women. Metastases occur in more than 70 % of breast cancer patients and lead to increased morbidity and mortality [1, 2]. Therefore, the mechanism of breast cancer invasion and metastasis has always been the research hotspot.

Among the genes associated with metastasis of breast cancer, tumor metastasis suppressor gene 1 (TMSG1) is a novel tumor metastasis suppressor gene which was firstly cloned by our laboratory from non-metastatic cancer cell variant of human prostate carcinoma (PC-3M-2B4) using messenger RNA differential display in 1999 [3] (GenBank accession number AF189062 [4]). Using sense and antisense complementary DNA (cDNA) transfection, we found that homo sapiens longevity assurance homologue 2 of yeast LAG1 (LASS2)/TMSG1 could significantly suppress the invasion ability of human prostate cancer cell line PC-3M, human pulmonary giant cell carcinoma cell line PG, and

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human breast cancer cell line MDA-MB-231 [5, 6–8]. Furthermore, the identification of the gene LASS2/TMSG1 was confirmed successively by other laboratories [9]. In September 2001, LASS2 (GenBank accession number AF177338), a previously unknown human homologue of the yeast longevity assurance gene LAG1, was identified from human liver cDNA library by the Cancer Research Institute of Shanghai, with nearly 100 % identical sequence to TMSG1 gene [10]. They 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: the high-affinity asialoglycoprotein receptors type 1 (AGPRH1) and asialoglycoprotein receptors type 2 (AGPRH2), the organic cation transporter-1 (OCT1), and the proteolipid subunit of vacuolar H⁺ ATPase (VPL), which suggested LASS2/TMSG1 was a membrane-association protein. In April 2008, Laviad found that there were two alternatively spliced transcripts of LASS2/TMSG1 gene encoding the same protein [11]. Furthermore, in 2012, our lab [6–8] 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 prostate cancer cell lines PC-3M-1E8 and PC-3M-2B4 cells. Therefore, TMSG1 gene is also named as LASS2, TRH3, SP260, Cers2, or LAG1Hs-2 (named as LASS2/TMSG1 below). LASS2/TMSG1 gene contains the conserved TLC domain and the Hox-like domain, and its encoded protein can synthesize very long acyl chain ceramides [12].

LASS2/TMSG1 belongs to the LASS family, which includes LASS1 to LASS6 [13]. The main function of the LASS family is involving the synthesis of ceramides, which play important roles in cell proliferation, apoptosis, and differentiation. Recently, it has been reported that expression levels of some LASS family members are altered in breast cancer [14]. However, the clinical significance of the altered expression of LASS2/TMSG1 in breast cancer is still unknown. In this study, we cloned a full length of 1.2 kb LASS2/TMSG1 cDNA coding for a protein of 380 amino acids, constructed pcDNA3 eukaryotic expression plasmid of LASS2/TMSG1, and then transfected it into human breast cancer cell line MCF-7 by lipofectin method to observe the biological effects and explore the molecular mechanism.

Materials and methods

Cells and cell culture

Human breast cancer cell lines MCF-7 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10 % quality fetal bovine serum (FBS, purchased from

HyClone, Logan, UT), 100 units/mL of penicillin, and 100 µg/mL of streptomycin at 37 °C in 5 % CO₂.

Construction of vector

The full-length coding region of human LASS2/TMSG1 gene was amplified from PC-3M-2B4 cDNA library by specific primers (sense: 5'-CGCGAATTCGCCGCCACCATGCTC CAGACCTTGTATGATTA-3'; antisense: 5'-CGCCTCGA GGTCATTCTTACGATGGTTGTTATT-3'). The PCR products were cloned into the eukaryotic expression vector pcDNA3 (Invitrogen) between the restriction enzyme sites (EcoRI and XhoI) with FLAG tag in the carboxyl terminus. The recombinant plasmid was transformed into *Escherichia coli*. Positive clones were selected and the plasmid was extracted by PureYield™ Plasmid Midprep extraction kit (Promega). Sequencing proved correct clone was used for the next procedure.

Transfection

Transfection of plasmid DNAs into MCF-7 cells was done using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Briefly, 1×10^5 MCF-7 cells were seeded per well into six-well plates (Corning, Inc., Corning, NY). The cells were transfected at the logarithmic growth phase by the mixture of 2 µg plasmid and 5 µL Lipofectamine 2000 in 1 mL medium without serum and antibiotics. The cells were incubated for 6 h, and then 1 mL medium containing 20 % serum was added into each well. The next day, the cells were transported into a new cell culture flask (Corning, Inc., Corning, NY) and were cultured and screened in the medium containing 10 % serum and 1.2 mg/mL G418 (Invitrogen) for at least 2 weeks until the non-transfected MCF7 cells cultured in the controlled wells were all killed. At least six clones of each cell line were selected and cultured separately. Two stable transfectants of LASS2/TMSG1 plasmid (named LASS2/TMSG1 group 1 and LASS2/TMSG1 group 2), and one stable transfectant of pcDNA3 plasmid (named control vector), were selected for the following tests.

Western blot analysis

Cells (5×10^5) were planted into 25 cm² cell culture dishes and cultured in HCO₃⁻-buffered 1640 medium (pH 7.0) containing 10 % FBS for 24 h. The supernatant was collected after 24 h culture. At the same time, the total cellular protein was extracted. The concentrations of collected protein samples were detected by the bicinchoninic acid assay (BCA) method (Pierce). Protein of 10 µg amount from each sample was added to each lane, separated in 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (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 anti-human-LASS2/TMSG1 antibody (1:2000, Santa Cruz) or polyclonal rabbit anti-human-matrix metalloproteinase-2 (MMP-2) antibody (1:1000, CST) or monoclonal mouse anti-human- β -actin (1:2500, Santa Cruz) as a control. The membranes were then washed and incubated for 2 h at room temperature in TBST and 5 % dry milk containing horseradish peroxidase-conjugated rabbit anti-goat or goat anti-rabbit or goat anti-mouse secondary antibody (1:5000, Santa Cruz), then washed and incubated for 5 min with SuperSignal West Femto 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 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4), 0.25 mol/L sucrose, 1 mmol/L dithiothreitol (DTT), 1 mmol/L phenylmethanesulfonyl fluoride (PMSF), 1 mmol/L ethylenediaminetetraacetic acid (EDTA), and 10 mmol/L KCl. After homogenization, the mixture was centrifuged at 700g for 2 min and then 10,000g for 15 min. Supernatant was collected. The pellet was resuspended in the medium, homogenized again, and centrifuged same as above. Supernatant was collected together and centrifuged at 80,000g 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 by the BCA method with a spectrophotometer. Microsome proteins of 100 mg amount were 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 a spectrophotometer by colorimetric assay according to the manufacturer's instruction (GENMED SCIENTIFICS INC. USA). Absorbency (340 nm) of samples was measured at 0 min, and the measurement was repeated per 5 min at least four times. The negative control sample (supplied by the GENMED kit) was also tested by the same procedure. 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 BCECF fluorescence probe (Sigma Chemical Co., St.

Louis, MO). The exponential phase cells were seeded in a 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 $\mu\text{mol/L}$ 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 $\mu\text{mol/L}$ BCECF with a series of pH buffered by HCO_3^- -pHe value was converted to the extracellular proton concentration [15].

Activity of matrix metalloproteinase-2 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 gels were then immersed into the staining solution (0.05 % Coomassie Blue R250, 10 % acetic acid, 30 % methanol) for a few minutes and decolorized (10 % acetic acid and 30 % methanol only) for several times until the clear bands can be seen. The gels were observed in the gel imaging analysis system (Bio-Rad).

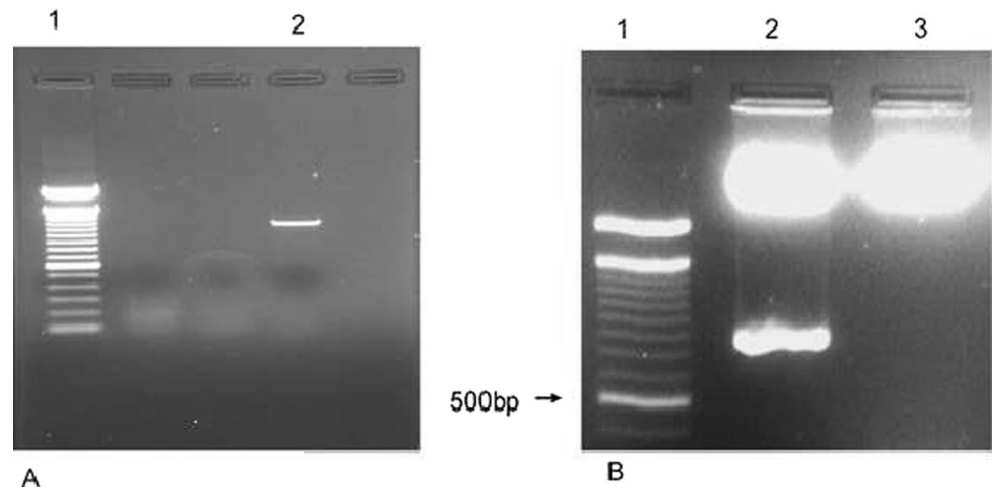
Cytoskeleton staining by rhodamine phalloidine

The stable transfected cells were seeded on slides in RPMI 1640 containing 10 % FBS. Twenty-four hours after attachment, the cells were fixed in acetone for 30 min and then rinsed with phosphate buffered saline (PBS) twice. The cells were treated with 0.1 % Triton X-100/PBS for 5 min at room temperature and incubated with rhodamine phalloidine (5 $\mu\text{g/mL}$, Sigma) for 30 min at room temperature. After rinse with PBS twice for 5 min, the slides were then mounted with mounting medium and examined by Leica TCS SP5 fluorescence microscope.

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. A 300-mesh nylon net was used to filter the cells and then 10 μL RNase was added, and the cells were incubated at 37 °C for 30 min. Cells

Fig. 1 Isolation of LASS2/TMSG1 and restriction mapping of recombinants. **a** The full length of 1.2 kb LASS2/TMSG1 cDNA was amplified from 2B4 cell by RT-PCR. **b** After BamH I digestion, fragments of plasmid were 0.878 and 5.7 kb. 1, 100 bp DNA marker; 2, pcDNA3-LASS2/TMSG1/BamH I; 3, pcDNA3-LASS2/TMSG1



were dyed by PI before detected by the flow cytometer (Becton, Dickinson and Company, BD).

Apoptosis analysis

Cells were digested by trypsin and washed twice by PBS. A 300-mesh nylon net was used to filter the cells. A 200 μ L amount of binding buffer and 10 μ L Annexin-V-FITC (Gene Research Center of Peking University) were used to make the resuspension of cells and cells were incubated at 4 $^{\circ}$ C for

30 min. Cells were dyed by 5 μ L PI and 300 μ L binding buffer before detected by the flow cytometer.

Cell proliferation assay

We cultured cells in a 24-well plate at a density of 5000 cells per well. Every clone was cultured continuously for 8 days, digested by 0.25 % trypsin, and counted for cell numbers every day. The experiment was repeated three times.

Fig. 2 **a, b** Western blot revealed 45 kDa of LASS2/TMSG1 protein expression was fivefold increased in the both LASS2/TMSG1-pcDNA3 groups compared with that in the control groups ($*P<0.05$)

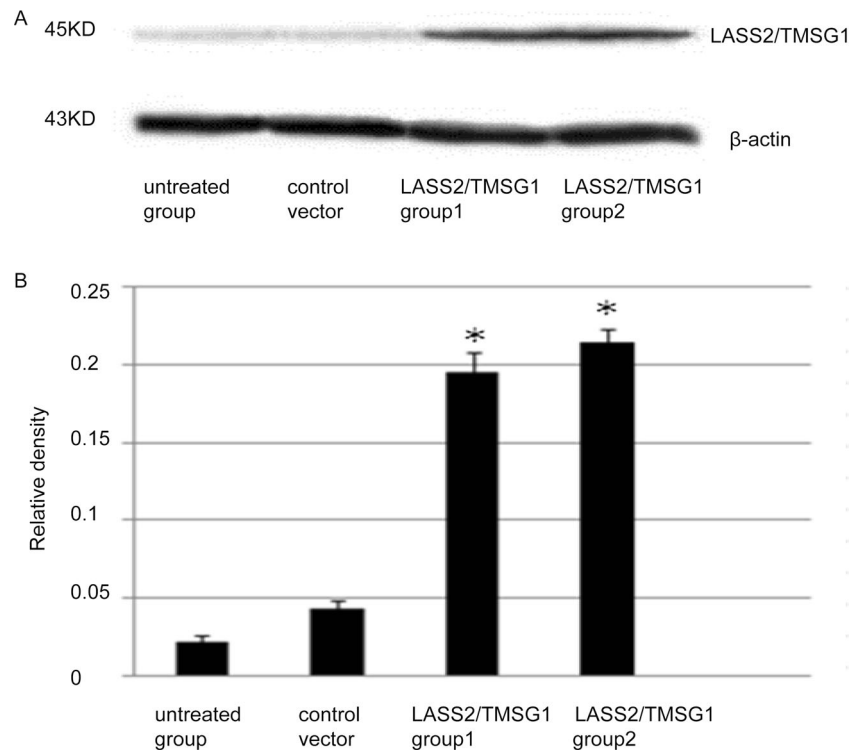


Table 1 V-ATPase activity of four groups

Cell lines	V-ATPase activity in the 10th min			Mean±SD (U/mg)
	First	Second	Third	
Untreated group	32.363	36.350	34.325	34.346±1.994
Control vector	30.418	31.897	32.588	31.635±1.109
LASS2/TMSG1 group 1	17.283	16.077	19.116	17.492±1.530*
LASS2/TMSG1 group 2	10.659	11.543	10.305	10.836±0.638*

* $P < 0.05$, $n = 3$

Soft agar assay

Cells 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.

In vitro cell migration

Into a six-well plate, 2.5×10^5 cells (400 μL) were added and incubated at 37 °C, 5 % CO_2 for 48 h. When the bottom of the well was covered by the monolayer of cells, the cells were scratched with 200 μL sterile gunpoint carefully, and the six-well plate was gently washed three times to remove cell debris with PBS. Fibronectin (16 $\mu\text{g}/\text{mL}$) was added into each well, cells were incubated at 37 °C, 5 % CO_2 incubator for 24 h, then they were observed under the microscope, and the rate of

Fig. 3 a, b The V-ATPase activity in LASS2/TMSG1-pcDNA3 cells significantly decreased compared with that of untreated cells and control vector cells (17.492±1.530 versus 31.635±1.109 U/mg of V-ATPase activity in the 10th min) (* $P < 0.05$, $n = 3$)

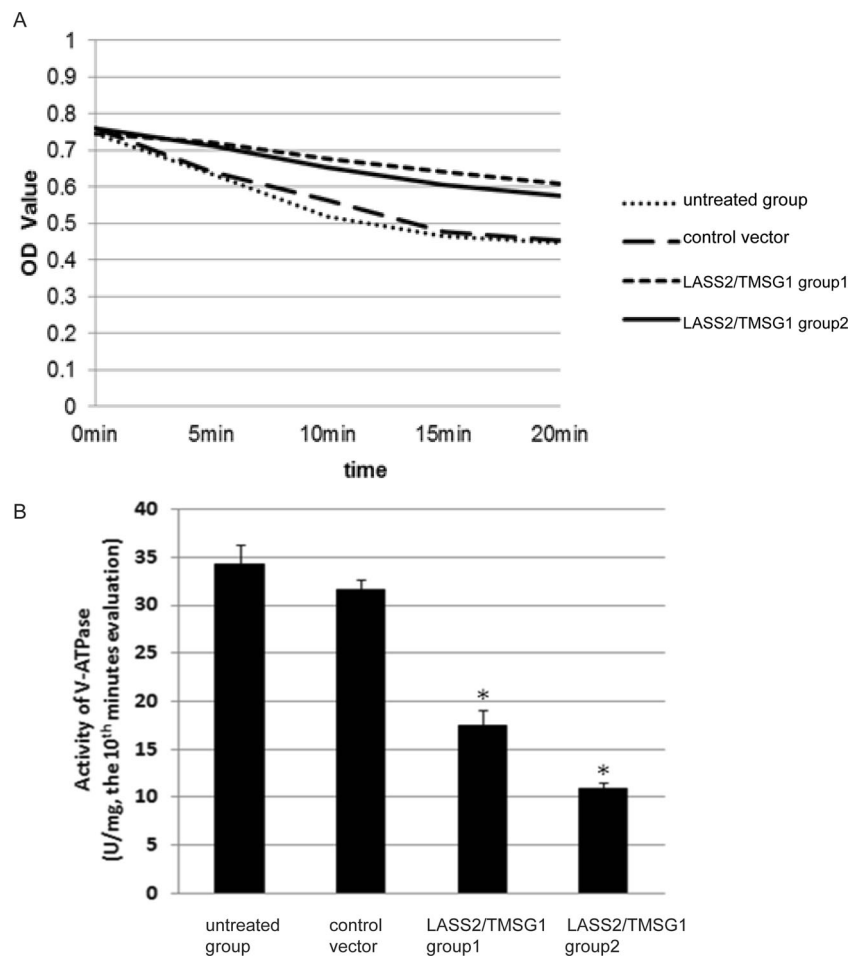
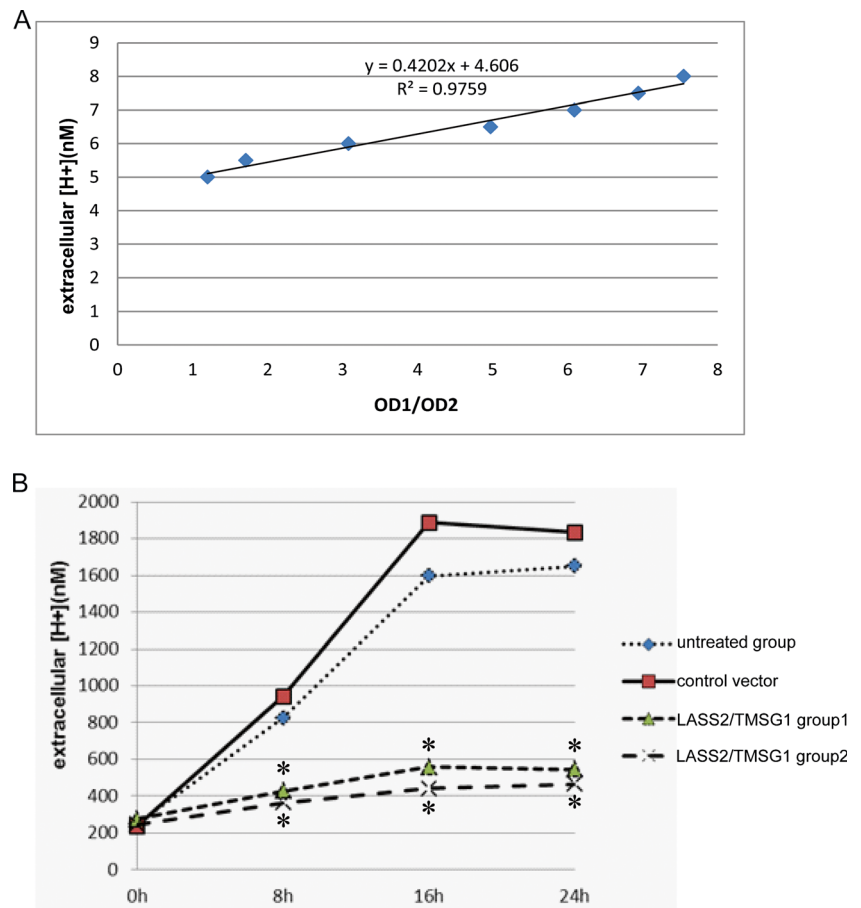


Fig. 4 a, b The concentration of extracellular hydrogen ion was measured by pH-sensitive BCECF. The proton secretion of LASS2/TMSG1-pcDNA3-treated cells was notably decreased from 8 h compared with that of untreated cells or control vector-treated cells ($P < 0.05$, $n = 3$)



wound healing was calculated finally. The experiment was repeated three times.

Invasion assay

On rehydrated Matrigel-coated culture inserts with 8- μ m-diameter pore size membranes (BD BioCoat™ Matrigel™ Invasion Chambers; BD Biosciences, Bedford, MA) in 24-transwell cell culture dishes, 2×10^5 cells (400 μ L) in exponential growth phase were planted. The bottom chamber contained 200 μ L of NIH 3T3-conditioned serum-free medium. After incubation for 12 h at 37 °C in a humidified 5 % CO₂ incubator, non-invaded cells remaining in the insert were removed with a cotton swab. Migrated cells on the bottom of the filters were stained with HE. Cells on the filters were counted under the microscope. The experiment was repeated three times.

Statistical analysis

Two-tailed Student's *t* test was used to analyze experiment data. $P < 0.05$ was considered to be of statistical difference.

Results

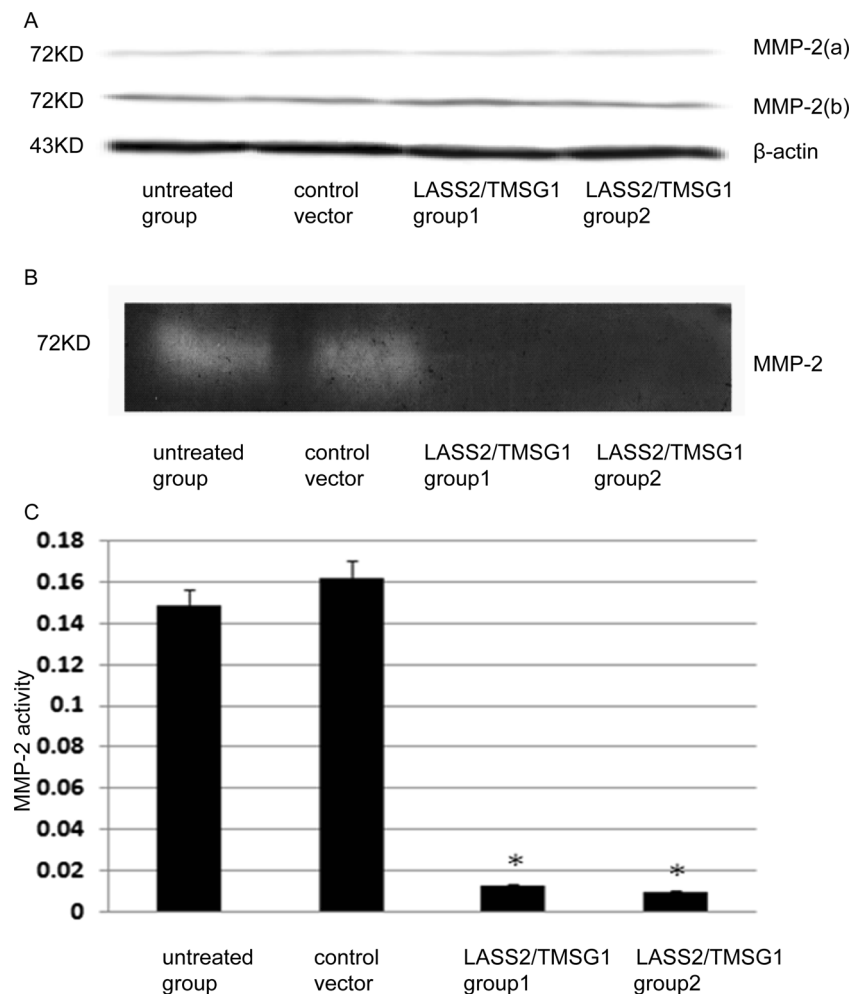
Isolation of LASS2/TMSG1 and identification of recombinants

The full length of 1.2 kb LASS2/TMSG1 cDNA with total open reading frame of 380 amino acids was amplified from PC-3M-2B4 cell by RT-PCR (Fig. 1a). Sequencing result of the cDNA products was correct. Recombinants were identified by agarose gel electrophoresis, and the plasmid size of about 6.6 kb was primarily regarded as positive clones. Recombinant DNA were restricted with *Bam*H I digestion and two bands were visual in agarose gel electrophoresis. The fragments of about 0.878 and 5.7 kb indicated the positive clone (Fig. 1b, lane 2). Positive recombinants were identified by sequence analysis.

Generation of breast cancer cell stably overexpressing LASS2/TMSG1

Human breast cancer cell lines MCF-7 were transfected with the constructed plasmid encoding LASS2/TMSG1-pcDNA3 and with negative controlled plasmid,

Fig. 5 The expression, secretion, and gelatinase activity of MMP-2 in four groups of MCF-7 cells. **a** MMP-2 protein in the MCF7 cells (**b**) and supernatant (**a**) was analyzed by Western blot, but there was no significant difference in the expression and secretion of MMP-2 protein among the LASS2/TMSG1 cells and the control cells. **b** and **c** the activity of MMP-2 were examined by gelatin zymography. The activity of MMP-2 in the supernatant of the LASS2/TMSG1-pcDNA3 cells revealed dramatic reduction of 94 % compared with the control cells ($P < 0.05$)



respectively. The stable transfectants with LASS2/TMSG1 and negative control were established by G418 screening for at least 2 weeks. The expression of LASS2/TMSG1 protein about 45 kDa was detected by Western blot, which revealed that LASS2/TMSG1-pcDNA3 increased fivefold more LASS2/TMSG1 production of that in the control cultures as shown in Fig. 2 ($P < 0.05$).

LASS2/TMSG1 decreases the activity of V-ATPase

The activity of V-ATPase was detected by GENMED's V-ATPase activity assay kit. The activity of V-ATPase = $(\Delta OD \text{ of sample} - \Delta OD \text{ of negative control}) \div (\text{concentration} \times 31.1) \times 10^3 \text{ U/mg}$. The results showed that the V-ATPase activity in LASS2/TMSG1-pcDNA3 cells decreased with statistical significance compared with control vector cells (17.492 ± 1.530 versus $31.635 \pm 1.109 \text{ U/mg}$ of V-ATPase activity in the 10th min) ($P < 0.05$, $n = 3$), as shown in Table 1 and Fig. 3.

LASS2/TMSG1 decreases extracellular H^+ concentration in MCF-7 cells

The fitted regression line was obtained via measurement of OD value in a PH-gradient culture solution. The proton secretion of LASS2/TMSG1-pcDNA3-treated cells was notably decreased from 8 h compared with that of untreated cells or control vector-treated cells ($P < 0.05$, $n = 3$, Fig. 4).

LASS2/TMSG1 decreases the activity of MMP-2 in the supernatant of the cultured cells

We detected the expression, secretion level, and activity of MMP-2 of the four group cells, which were closely related to cancer metastasis according to the previous reports. Western blot showed that there was no significant difference in the expression and secretion of MMP-2 among the LASS2/TMSG1-pcDNA3 cells and control cells (Fig. 5a) in total protein and cell supernatant of the MCF-7 cells, indicating that the LASS2/TMSG1 did not affect the expression and secretion of MMP-2 protein.

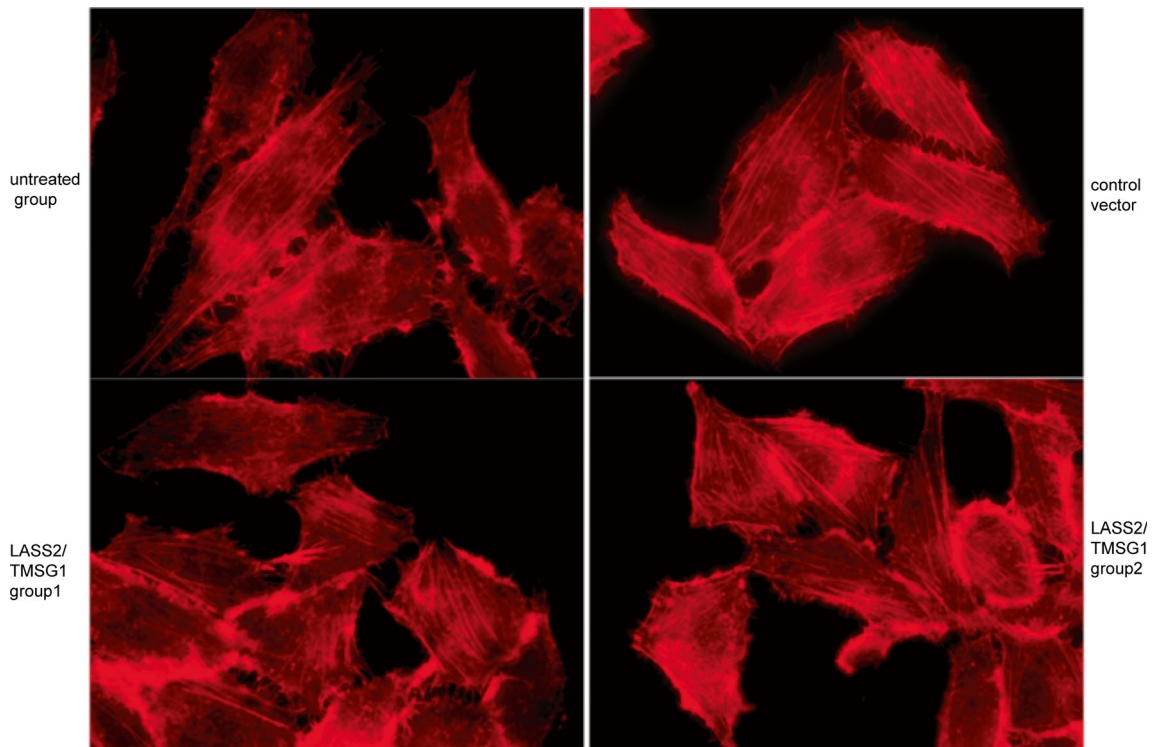


Fig. 6 There was no obvious difference in the morphology and distribution of cytoskeleton among the four groups by staining of rhodamine phalloidine

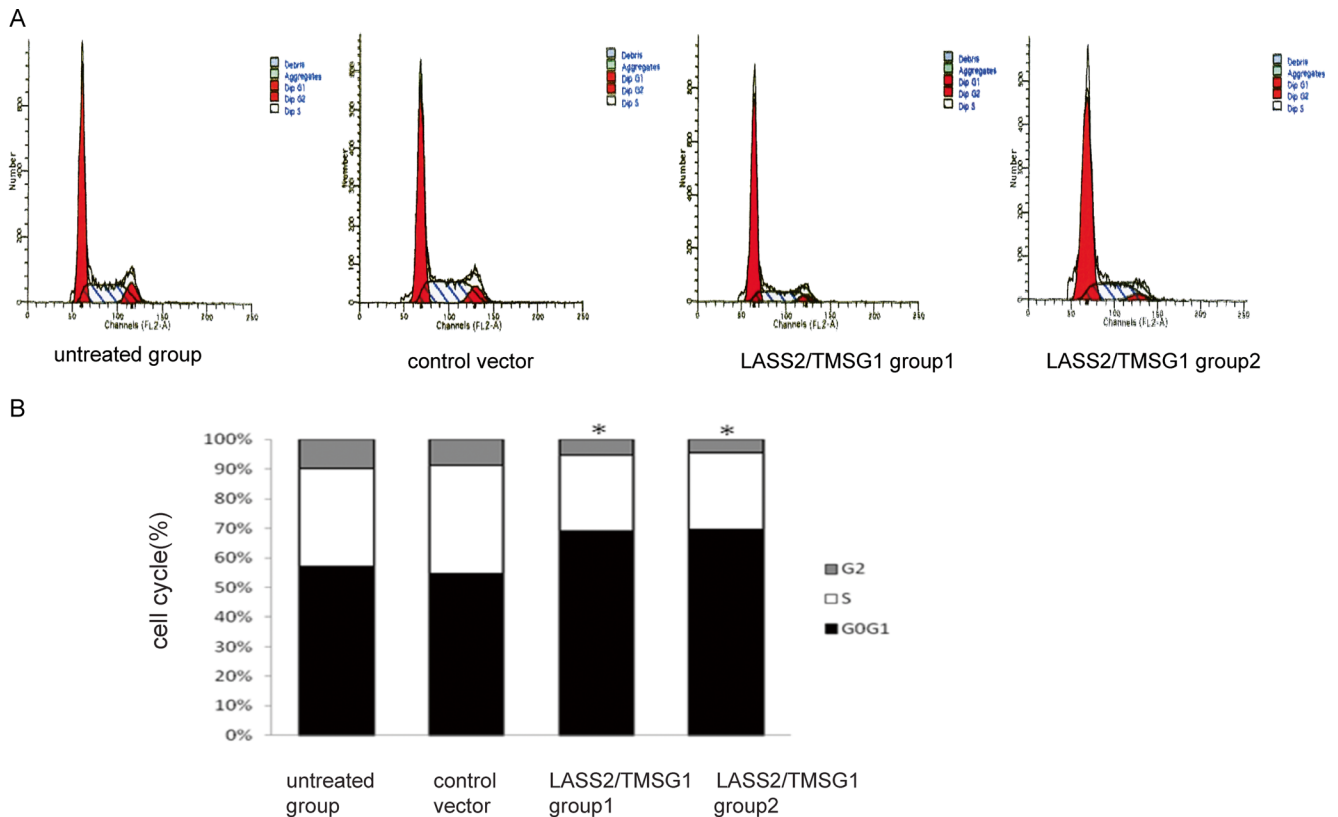


Fig. 7 a, b Flow cytometry analysis showed that the peak height of phase S and G2 in the LASS2/TMSG1-overexpression cells was lower than that in untreated or control cells which indicated an apparent decline of the percentage of S/G2 cells and an obvious increase of the percentage of cells arrested in G0/G1 phase from 54.7 to 69.5 % (* $P < 0.05$, $n = 3$)

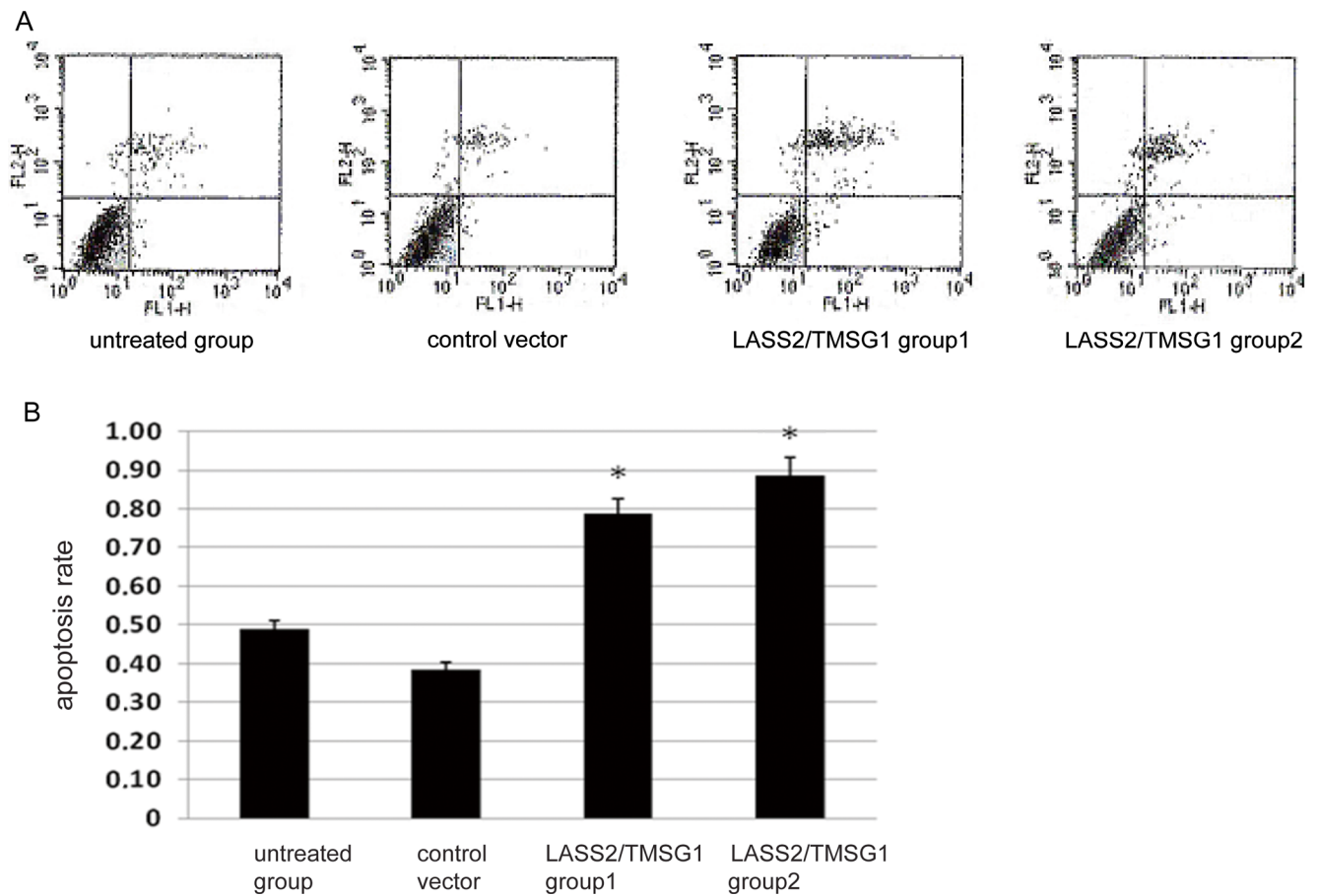


Fig. 8 a, b Flow cytometry showed LASS2/TMSG1-pcDNA3 groups significantly increased the apoptosis rate relative to the MCF-7 control cell lines. The apoptosis rate in untreated cells, control vector cells, LASS2/TMSG1 group 1, and LASS2/TMSG1 group 2 were 4.87 ± 0.58 , 3.85 ± 0.94 , 7.88 ± 0.85 , and 8.88 ± 0.89 %, respectively ($*P < 0.05$, $n = 3$)

Furthermore, the supernatant of cultured cells was collected and the gelatinase activity was assayed with gelatin zymography. The activity of MMP-2 in the supernatant of the LASS2/TMSG1-pcDNA3 cells revealed dramatic reduction of 94 % compared with the control cells (Fig. 5b, c), which indicated that the LASS2/TMSG1 decreased the activity of MMP-2 protein.

LASS2/TMSG1 has no effect on the morphology and distribution of cytoskeleton

There was no obvious difference in the morphology and distribution of cytoskeleton among the four groups by staining of rhodamine phalloidine (Fig. 6).

Fig. 9 LASS2/TMSG1 overexpression groups were in dramatic growth suppression compared with control cells ($*P < 0.05$). This effect was more pronounced after the fourth day

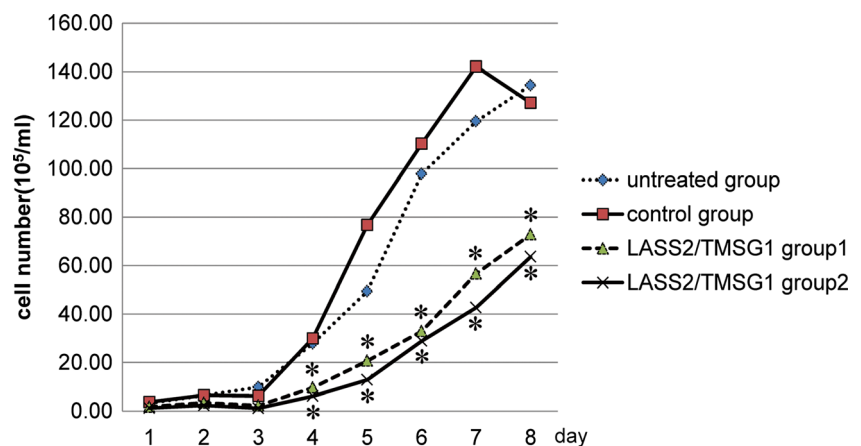
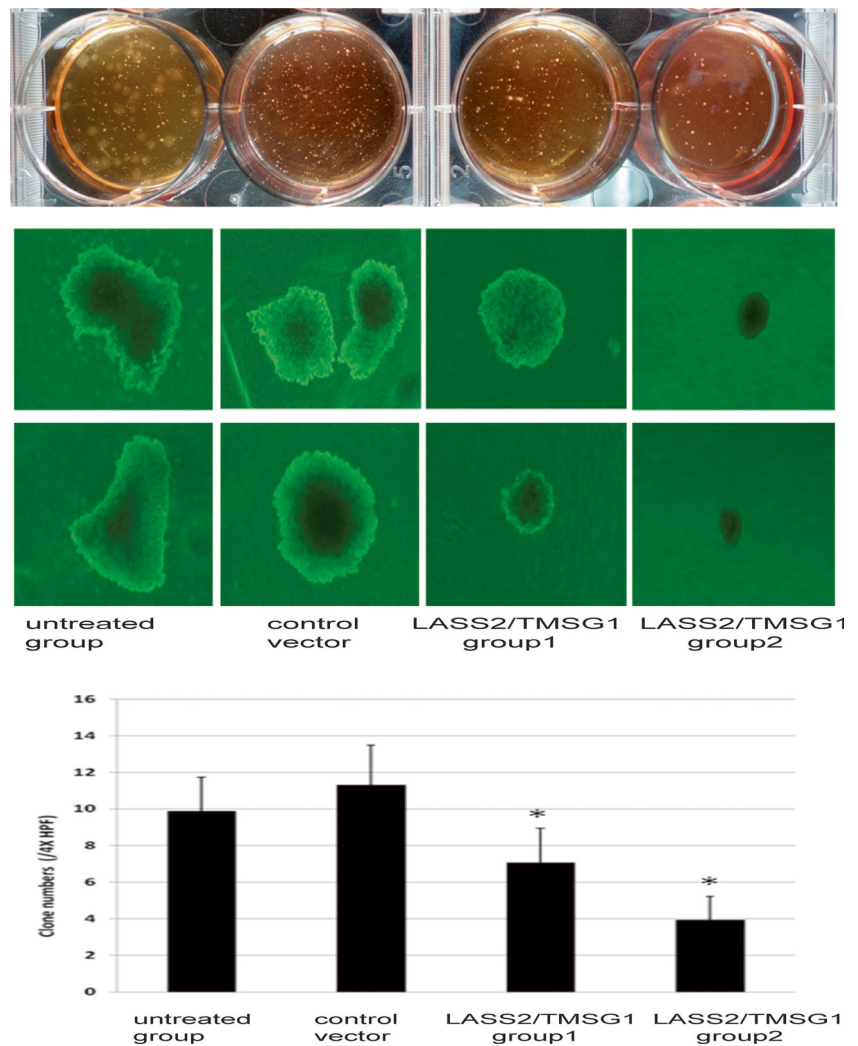


Fig. 10 Using soft agar assay, the LASS2/TMSG1-pcDNA3 cells produced less colonies relative to the untransfected MCF-7 cells and control vector cells after 4 weeks culture. The colony numbers of untreated cells, control vector cells, LASS2/TMSG1 group 1, and LASS2/TMSG1 group 2 in soft agar were 9.87 ± 1.85 , 11.3 ± 2.19 , 7.07 ± 1.87 and 3.93 ± 1.28 , respectively ($*P < 0.05$, upper and lower). Furthermore, the colony diameter of untreated and control vector groups was greatly larger and more irregular than that of LASS2/TMSG1-pcDNA3 groups (*middle*)



LASS2/TMSG1 had great effects on cell cycle distribution

The analysis of cell-cycle distribution revealed that the over-expression of LASS2/TMSG1 had great effect on cell-cycle progression. Flow cytometry analysis showed that the peak height of phase S and G2 in the LASS2/TMSG1 overexpression cells was lower than that in untreated or control cells which indicated an apparent decline of the percentage of S/G2 cells and an obvious increase of the percentage of cells arrested in G0/G1 phase from 54.7 to 69.5 % ($P < 0.05$, $n = 3$, Fig. 7), suggesting that the LASS2/TMSG1 might induce G0/G1 retardation and result in reduction of cell proliferation.

LASS2/TMSG1 increased the apoptosis rate of MCF-7 cells

The effect of apoptosis rate by LASS2/TMSG1-pcDNA3 on MCF-7 cells was further evaluated using flow cytometry analysis. The apoptosis rate in untreated cells, control vector cells, LASS2/TMSG1 group 1, and LASS2/TMSG1 group 2 were 4.87 ± 0.58 , 3.85 ± 0.94 , 7.88 ± 0.85 , and 8.88 ± 0.89 %, respectively, suggesting that the apoptosis rate significantly increased in both LASS2/TMSG1 groups ($P < 0.05$, $n = 3$, Fig. 8).

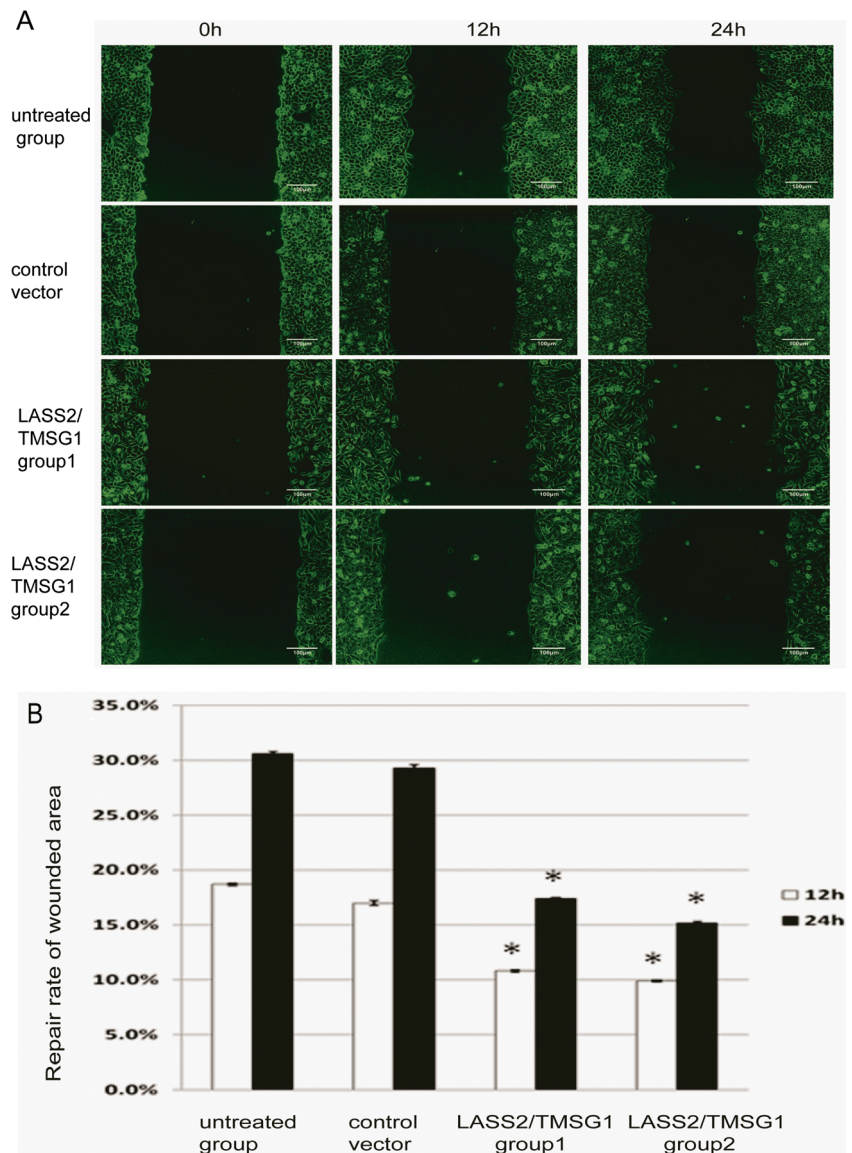
LASS2/TMSG1 suppressed cell growth in MCF-7 cells

The cell proliferation assay demonstrated that LASS2/TMSG1 overexpression caused to dramatic growth suppression of MCF-7 cells, as compared with control cells ($P < 0.05$, Fig. 9). This effect was more pronounced after the fourth day.

LASS2/TMSG1 decreased the colony-formation capacity of MCF7 cells

Using soft agar assay, the LASS2/TMSG1-pcDNA3 cells produced less colonies relative to the untransfected MCF-7 cells and control vector cells after 4 weeks culture. The colony numbers of untreated cells, control vector cells, LASS2/TMSG1 group 1, and LASS2/TMSG1 group 2 in soft agar were 9.87 ± 1.85 , 11.3 ± 2.19 , 7.07 ± 1.87 , and 3.93 ± 1.28 ,

Fig. 11 a, b The healing rate of LASS2/TMSG1-pcDNA3 cells was much slower than the control cells. The repair rate of wounded area in untreated cells, control vector cells, LASS2/TMSG1 group 1, and LASS2/TMSG1 group 2 were 30.6 ± 0.2 , 29.3 ± 0.3 , 17.4 ± 0.1 , and 15.2 ± 0.1 %, respectively ($*P<0.05$, $n=3$)



respectively, with statistically significant difference ($P<0.05$, $n=3$; Fig. 10a, c). Furthermore, the colony diameters of untreated and control vector groups was greatly larger and more irregular than those of LASS2/TMSG1-pcDNA3 groups (Fig. 10b). Therefore, LASS2/TMSG1 significantly decreased the colony-formation capacity of MCF-7 cell.

LASS2/TMSG1 suppressed the migration and invasion of MCF-7 cells

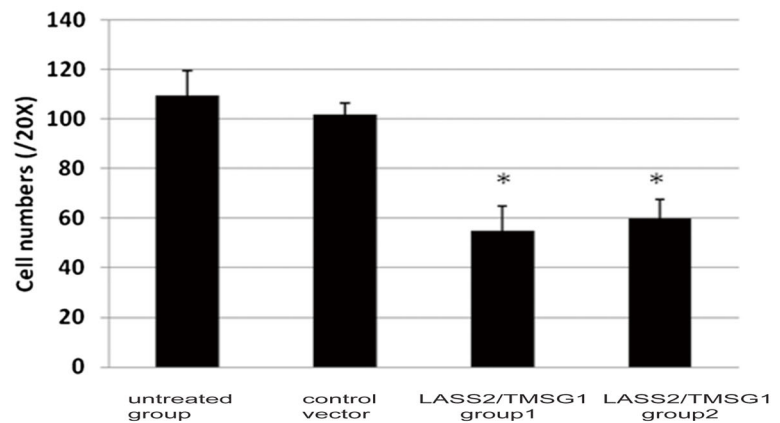
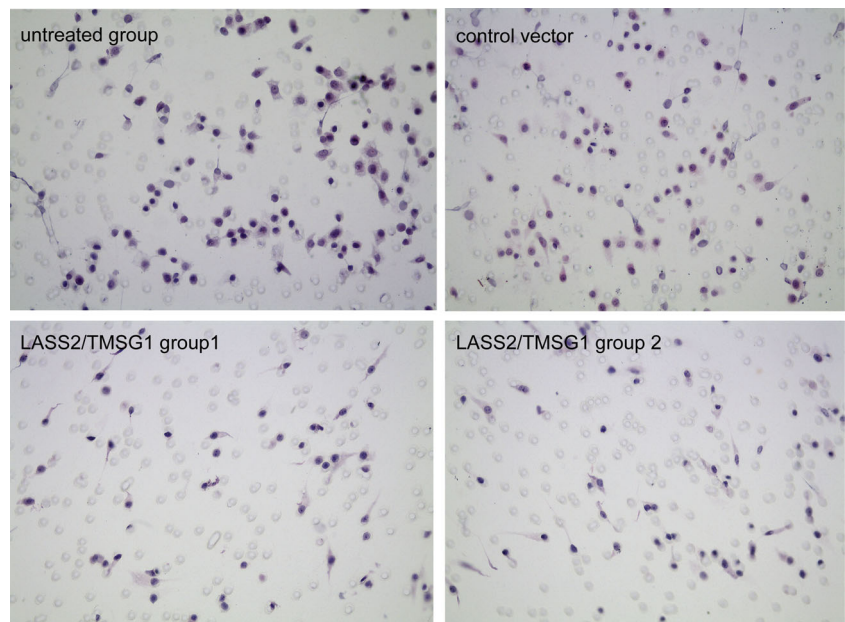
In wound migration assay, the cell-free wound gaps of monolayer healed more slowly in LASS2/TMSG1-pc DNA3 cells than in control cells. The repair rate of wounded area in untreated cells, control vector cells, LASS2/TMSG1 group 1, and LASS2/TMSG1 group 2 were 30.6 ± 0.2 , 29.3 ± 0.3 , 17.4 ± 0.1 , and 15.2 ± 0.1 %, respectively, with statistically significant difference ($P<0.05$, $n=3$; Fig. 11).

Furthermore, using the Boyden chamber invasion assay, the LASS2/TMSG1 overexpression cells displayed dramatically decreased invasion ability compared with the control cells. The cell numbers of invasion in untreated cells, control vector cells, LASS2/TMSG1 group 1, and LASS2/TMSG1 group 2 were 109.43 ± 10.05 , 102.00 ± 4.28 , 55.00 ± 10.07 , and 60.14 ± 7.65 , respectively ($P<0.05$, $n=3$; Fig. 12). Therefore, LASS2/TMSG1 significantly suppressed the migration and invasion ability of MCF-7 cells.

Discussion

Evidences are accumulating that acidic tumor microenvironment is involved in cancer progression. Among the key regulators of the acidic tumor microenvironment, V-ATPases

Fig. 12 Using Boyden chamber invasion assay, the LASS2/TMSG1-pcDNA3 cells displayed dramatically decreased invasion ability compared with the control cells. The cell numbers of invasion in untreated cells, control vector cells, LASS2/TMSG1 group 1, and LASS2/TMSG1 group 2 were 109.43 ± 10.05 , 102.00 ± 4.28 , 55.00 ± 10.07 , and 60.14 ± 7.65 , respectively ($*P < 0.05$, $n = 3$)



play an important role. In previous study, we reported 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 prostate cancer cell lines PC-3M-1E8 and PC-3M-2B4 cells [6–8]. However, the precise molecular mechanisms of LASS2/TMSG1 on cancer invasion and metastasis through V-ATPase in breast cancer are still unclear. Therefore, we cloned a full length of 1.2 kb LASS2/TMSG1 cDNA coding for a protein of 380 amino acids, reconstructed with pcDNA3 eukaryotic expression plasmids of LASS2/TMSG1, and transfected it into human breast cancer cell line MCF-7 using lipofectin method to observe the biological effects and explore the molecular mechanism.

In our experiments, we successfully established two MCF-7 cell lines stably overexpressing LASS2/TMSG1 protein. The expression of LASS2/TMSG1 protein about

45 kDa was detected by Western blot, which revealed that LASS2/TMSG1-pcDNA3 increased fivefold more LASS2/TMSG1 production of that in the untreated MCF-7 cells or in the control vector cells which only trace amounts of endogenous LASS2/TMSG1 could be detected. Curiously, the exogenous LASS2/TMSG1 protein in human breast cancer cell line MCF-7 was obviously less than that in human prostate cancer cell line PC-3M-2B4 with similar indolent biological behavior. In MCF-7 cells, the expression of LASS2/TMSG1 was distinctly lower than that of β -actin which was opposite to that in PC-3M-2B4 cells, suggesting there was possibly different regulatory mechanism of LASS2/TMSG1 in different tissues or cells.

Our experiments verified that LASS2/TMSG1 induced dramatic deceleration of proliferation and reduction of migration or invasion ability in MCF-7 cells. Therefore, it is

necessary to explore the molecular mechanism of LASS2/TMSG1 on tumor microenvironment and invasion. Furthermore, flow cytometry analysis showed that LASS2/TMSG1 induced G0/G1 retardation in MCF-7 cells and resulted in reduction of cell proliferation. The inhibition of proliferation by LASS2/TMSG1 was speculated to have a close relative with ceramide because LASS2/TMSG1 which is also named as ceramide synthase 2 (CerS2) and synthesizes very long acyl chain ceramides [16]. Ceramide is a key intermediate in the pathway of sphingolipid biosynthesis and is an important intracellular messenger that plays important roles in different cellular processes such as apoptosis, differentiation, and cell cycle [17]. CerS2 has been reported to induce cell cycle arrest at G0/G1 and Rb protein dephosphorylation in multiple cell lines. Our findings are in agreement with these publications. Ceramide is also a bioactive sphingolipid that mediates cell death [18]. Our data defined that LASS2/TMSG1 increased the apoptosis rate significantly in MCF-7 cells from 3.85 ± 0.94 to 7.88 ± 0.85 %, which might be related with increasing synthesis of ceramide. In breast cancer, LASS2/TMSG1 acts as a proapoptotic protein that increases chemosensitivity and inhibits tumor growth [19]. Upon apoptosis induction, CerS2 activity is stimulated at the mitochondria and mitochondria-associated membrane (MAMs), a distinct membrane compartment that links the endoplasmic reticulum to the mitochondria. Ceramide production is necessary for Bcl2-associated X protein (Bax) to insert into the mitochondrial membranes, oligomerize, and subsequently form a pore resulting in mitochondrial outer membrane permeabilization (MOMP) and cytochrome C release [20].

Moreover, in view of our experimental results, we speculated LASS2/TMSG1 may decrease the activity of V-ATPase and increase intracellular H^+ of MCF-7 cells via the direct interaction with V-ATPase and thereby induce cell apoptosis and inhibit cell proliferation. V-ATPase can also regulate the sensitivity of cells to these molecular signals by efficiently recycling of membrane receptor 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 [21]. In 2010, Tang et al. [22] 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, our lab [8] 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 prostate cancer cell lines PC-3M-1E8 and PC-3M-2B4 cells. Their interaction could play important roles in the apoptosis of tumor cells.

There are a great diversity of molecular mechanism of tumor invasion and metastasis, such as the formation of tumor chemokine, the activation of secreted MMP, rearrangement of

cytoskeleton, and so on. Our study revealed that overexpression of LASS2/TMSG1 protein can inhibit invasion of human breast cancer cell line MCF-7 in vitro through the decrease of the V-ATPase activity, suppress the proton transmembrane secretion, and thus decrease extracellular hydrogen ion concentration, and in turn the inactivation of secreted MMP-2 and non-degradation of ECM, which was in accordance with our previous study [6, 7]. The promoting effect of V-ATPase on cancer invasion and metastasis mainly relies on its maintaining acidic pH of extracellular microenvironment and very acidic luminal pH [21], 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 [23, 24, 25] and MMPs [26, 27].

The rearrangement of cytoskeleton is also an important mechanism of tumor invasion and metastasis, especially actin [28]. Our experiment showed there was no obvious difference in the morphology and distribution of cytoskeleton among the four groups by staining of rhodamine phalloidine, suggesting the rearrangement of cytoskeleton was not involved in LASS2/TMSG1's anti-invasion effect.

In conclusion, overexpression of LASS2/TMSG1 can inhibit invasion of human breast cancer cell line MCF-7 in vitro through the decrease of the V-ATPase activity and extracellular hydrogen ion concentration and, in turn, the inactivation of secreted MMP-2 and non-degradation of ECM, which ultimately suppressed tumor's invasion, indicating that LASS2/TMSG1 is a novel tumor metastasis suppressor gene and the regulation of LASS2/TMSG1 has potential for future cancer therapy.

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