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Silencing of a Novel Tumor Metastasis Suppressor Gene LASS2/TMSG1 Promotes Invasion of Prostate Cancer Cell In Vitro Through Increase of Vacuolar ATPase Activity

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ABSTRACT

Homo sapiens longevity assurance homologue 2 of yeast LAG1 (LASS2), also known as tumor metastasis suppressor gene 1 (TMSG1), is a newly found tumor metastasis suppressor gene in 1999. Preliminary studies showed that it not only suppressed tumor growth but also closely related to tumor metastasis, however, its molecular mechanisms is still unclear. There have been reported that protein encoded by LASS2/TMSG-1 could directly interact with the C subunit of Vacuolar ATPase (V-ATPase), which suggested that LASS2/TMSG1 might inhibit the invasion and metastasis through regulating the function of V-ATPase. Thus, in this study, we explored the effect of small interference RNA (siRNA) targeting LASS2/TMSG1 on the invasion of human prostate carcinoma cell line PC-3M-2B4 and its molecular mechanisms associated with the V-ATPase. Real-time fluorogentic quantitative PCR (RFQ-PCR) and Western blot revealed dramatic reduction of 84.5% and 60% in the levels of LASS2/TMSG1 mRNA and protein after transfection of siRNA in PC-3M-2B4 cells. The V-ATPase activity and extracellular hydrogen ion concentration were significantly increased in 2B4 cells transfected with the LASS2/TMSG1-siRNA compared with the controls. The activity of secreted MMP-2 was up-regulated in LASS2/TMSG1-siRNA treated cells compared with the controls; and the capacity for migration and invasion in LASS2/TMSG1-siRNA treated cells was significantly higher than the controls. Thus, we concluded that silencing of LASS2/TMSG1 may promote invasion of prostate cancer cell in vitro through increase of V-ATPase activity and extracellular hydrogen ion concentration and in turn the activation of secreted MMP-2. J. Cell. Biochem. 113: 2356–2363, 2012.

KEY WORDS: SIRNA; LASS2/TMSG1; PROSTATE CANCER; V-ATPASE; MMP-2

Prostate cancer is the principal malignant tumor threatening the health of senile male all over the world at present. Meanwhile, it is estimated that prostate cancer accounts for about 33% (220,900) of new cancer cases and about 28,900 Americans will die from prostate cancer every year, which is the second most common fatal cancer among men [Jemal et al., 2005]. In China, the morbidity of prostate cancer is obviously lower than that in the USA, however, with the extending of life span, the change on food structure and the improvement of diagnosis technique in recent years, the incidence of prostate cancer is rapidly increasing and it is becoming the third most common cancer of male urogenital system in China. In the Beijing population, the incidence was 1.9 persons/10 million in 1983, but it increased to 21.08 persons/10 million in 2003 [Gu, 2003]. Furthermore, most patients with prostate cancer are dying of tumor metastasis and the mortality is about 11%, therefore, it is most urgent to study the metastasis mechanism of prostate cancer and explore the genes and treatment target associated with the metastasis of prostate cancer.

Among the genes associated with metastasis of prostate carcinoma, TMSG1 gene is a novel tumor metastasis suppressor gene which was firstly cloned by our laboratory from non-metastatic and metastatic cancer cell variants of human prostate carcinoma using mRNA differential display in 1999 [Liu et al., 1999] (GenBank accession number AF189062 [Ma et al., 2002]). Furthermore, the identification of the gene was confirmed successively by other laboratories [Pan et al., 2001; Strausberg et al., 2002; Laviad et al., 2008]. In September 2001, homo sapiens longevity assurance homologue 2 of yeast LAG1 (LASS2; Genbank

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accession no. AF177338), a previously unknown human homologue of the yeast longevity assurance gene LAG1, was identified from human liver cDNA library by cancer research institute of Shanghai, with nearly 100% identical sequence to TMSG1 gene [Pan et al., 2001]. Therefore, LASS2/TMSG1 gene is also named as TRH3 gene, SP260 gene, CerS2 gene, or LAG1Hs-2. It contains the conserved TLC domain and the Hox-like domain, and synthesizes very long acylchain ceramides.

Meanwhile, it was found that protein encoded by LASS2/TMSG-1 could directly interact with the C subunit of V-ATPase [Pan et al., 2001; Sennoune et al., 2004a], which suggested that LASS2/TMSG1 might inhibit the invasion and metastasis through regulating the function of V-ATPase. V-ATPase (Vacuolar-Type (H^+)-ATPases) are ubiquitous proton-translocating pumps of eukaryotic cells. The pumps are located in membranes of vacuoles, lysosomes, and other components of the endomembrane system, as well as in certain specialized plasma membranes. Protons are pumped out of the cytoplasm into the organelle or the extracellular space in order to regulate the intracellular acidity. The C subunit of V-ATPase plays an important role in structure and formation of the protons channel. V-ATPase-mediated regulation of pHi has also been implicated in cancer drug resistance and the regulation of apoptosis [Sennoune et al., 2004b]. Moreover, V-ATPase is expressed in plasma membrane of some tumor cells, which is correlated with the metastatic potential of these cells. In vitro, the transfected fibroblast cell line with overexpressing the C subunit of V-ATPase behaves with increased invasion accompanied by up-regulation of secretion of MMP-2 [Lu et al., 2005]. MMP-2 is closely related to cancer metastasis, and its activity is increased at the invasive borders of the metastatic tumor tissue of hepatocellular carcinoma, colon carcinoma, or pancreatic carcinoma. However, the molecular mechanism by which LASS2/TMSG1 on cancer metastasis through V-ATPase is unclear at present.

Therefore, in this study, to further investigate the molecular mechanisms of LASS2/TMSG1 on metastasis of prostate cancer, small interfering RNAs (siRNAs) targeting LASS2/TMSG1 will be adopted to silence the gene expression in human prostate cancer cell line PC-3M-2B4 (low metastatic potential, high expression of LASS2/TMSG1) [Ma et al., 2002] in order to measure the -ATPase activity and extracellular hydrogen ion concentration before and after interference, analyze the expression and activity of the matrix metalloproteinase-2(MMP-2) protein in the supernatant and cells and observe the migration and invasion of PC-3M-2B4 cells in vitro.

MATERIALS AND METHODS

CELLS AND CELL CULTURE

Human prostate cancer cell lines PC-3M-2B4 cells [Liu et al., 1999] (non-metastatic cancer variant from human prostate carcinoma 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. Cells were cultured in 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% CO_2.

CONSTRUCTION OF siRNAs

We designed and purchased four different siRNA duplexes of LASS2/TMSG1 (Genbank accession nos. NM013384, NM181746, and NM022075) from Qiagen Company (USA). Nucleotides 1255–1275 of the human LASS2/TMSG1 cDNA (5′-TGCGCTATAGGGTCACTTTAA-3′, Genbank accession no. NM013384) were used as the target sequence of siRNA-1; nucleotides 722–742 of the human LASS2/TMSG1 cDNA (5′-CCGGCCCAGTCTCCTCAAGAA-3′, Genbank accession no. NM022075) were used as the target sequence of siRNA-2; nucleotides 1175–1195 of the human LASS2/TMSG1 cDNA (5′-AACCATCGTAAGAATGACTGA-3′, Genbank accession no. NM013384) were used as the target sequence of siRNA-3; nucleotides 502–522 of the human LASS2/TMSG1 cDNA (5′-CATGGCCGTCATTGTGGATAA-3′, Genbank accession no. NM013384) were used as the target sequence of siRNA-4. The non-specific control siRNA duplexes (AllStar Negative Control siRNA) were also purchased from the Qiagen Company.

TRANSFECTION

Transfection of siRNA targeting LASS2/TMSG1 and AllStars Negative Control siRNA into PC-3M-2B4 cells was done using Lipofectamine^{TM}2000 (Invitrogen, Carlsbad, CA). Briefly, 3×10^5 cells were seeded per well into six-well plates (Corning, Inc., Corning, NY). The cells were transfected using 0.1 nmol siRNA-1, siRNA-2, siRNA-3, siRNA-4, AllStars Negative Control siRNA (20 μmol/L) mixed with 5 μl Lipofectamine^{TM}2000 in 500 μl medium without serum and antibiotics, then was added into each well. Simultaneously, PC-3M-2B4 cells were treated as blank control with PBS. After 4–6 h incubation, the culture medium was disposed and the new RPMI1640 culture medium containing 10% fetal bovine serum was added to cultivate for 24 h. The experiment was done in triplicates. More than nine wells were treated with the same kind of siRNA.

RNA ISOLATION, REAL-TIME FLUOROGENTIC QUANTITATIVE PCR (RFQ-PCR)

Total RNA from cultured cells was isolated using the TRIzol reagent (Invitrogen), and first-strand cDNA was synthesized using 2 μg total RNA and TransScript First-Strand cDNA Synthesis SuperMix (Transgene, Beijing). In accordance with TransStart Green qPCR SuperMix kit instructions (Transgene), PCR reaction was done. Primers of GADPH and LASS2/TMSG1 were searched by Gene Bank, designed by use of Primer 5.0 software and synthesized by Augct biological Company in Beijing. The LASS2/TMSG1 or GADPH was amplified by RFQ-PCR at conditions of 95°C for 3 min; followed 45 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s in the presence of TransStart Green qPCR SuperMix (Transgene), 10 pmol/μl of forward 5′-TCCTGCCTTCTTTGGCTATTACTT-3′ and reverse 5′-TGCGTTCATCTTCTACCAGCTTTC-3′ primers for LASS2/TMSG1, or 10 pmol/μl of forward 5′-GAAGGTGAAGGTCGGAGTC-3′ and

reverse 5′-GAAGATGGTGATGGGATTTC-3′ primers for GADPH. The experiment was repeated three times.

EXPRESSION AND SECRETION OF LASS2/TMSG1 AND MATRIX METALLOPROTEINASE-2 BY WESTERN BLOT

The expression and secretion of LASS2/TMSG1 and MMP-2 in cells was assayed by Western blot. After 24-h transfection, cells of three groups were digested and transferred to 35 mm Petri dishes (3×10^5 cells/dish). After cells adhesion, the old culture medium was disposed and then 500-μl serum-free RPMI 1640 culture medium was added into the cells. 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 microgram of protein from each sample were 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 anti-human-LASS2/TMSG1 antibody (1:2,000, Santa Cruz) or rabbit anti-human-MMP-2 antibody (1:1,000, CST), or monoclonal mouse anti-human-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 secondary antibody (1:5,000, Santa Cruz), washed and incubated for 5 min with Super Signal West Femoto Maximum Sensitivity Substrate (Pierce), and then exposed to X-ray films.

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 gel was observed on gel imaging analysis system.

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 the same as above. Supernatant was collected together and centrifuged at 80,000*g* for 1 h 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. 100 mg microsome proteins 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/LEDTA, 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., USA). Absorption (340 nm) of samples at 0 min, the 5th minute, and the 10th minute 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). The cells in the exponential phase were seeded in six-well plate for 12 h, siRNA targeting LASS2/TMSG1 and AllStars Negative Control siRNA were transfected into six-well plate. After 24-h transfection, cell culture medium was replaced 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. The medium was removed after the cells attached to the plate. 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. Absorption (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].

INVASION CHAMBER 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, non-migrated cells remaining in the insert were removed with a cotton swab. Migrated cells on the bottom of the filters were stained with HE for 30 min. Cells on the filters were counted using a microscope. Triplicate filters were counted in each experiment.

IN VITRO CELL MIGRATION

2.5×10^5 cells (400 μl) were added into six-well plate 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 μg/ml) was added into each well, cells were incubated at 37°C, 5% CO_2 incubator for 24 h, then they were observed under fluorescence microscope camera, and the cell number of migration was calculated finally. The experiment was repeated three times.

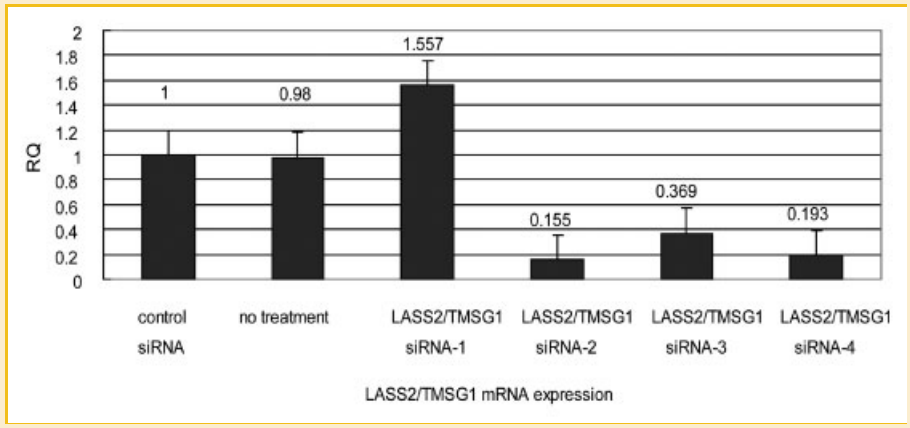

Fig. 1. Expression of LASS2/TMSG1 mRNA after transfection with different siRNA fragments in PC-3M-2B4 cells by RFQ-PCR (n = 3). RFQ-PCR revealed dramatic reduction of 84.5% with siRNA-2, 63.1% with siRNA-3, and 80.7% with siRNA-4 in the levels of LASS2/TMSG1 mRNA after transfection of siRNA in PC-3M-2B4 cells, compared with AllStar Negative Control siRNA or the untransfected cultures ($P < 0.05$). RQ: relative quantity (% of the control).

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

EFFECTS OF LASS2/TMSG1 siRNAs ON THE EXPRESSION OF LASS2/TMSG1 FROM PC-3M-2B4 CELLS

We examined four LASS2/TMSG1 siRNAs to target human LASS2/TMSG1 as described above. RFQ-PCR analysis of LASS2/TMSG1 of the siRNA-transfected PC-3M-2B4 cells showed that siRNA-2 effectively blocked the expression of LASS2/TMSG1 mRNA. RFQ-PCR revealed dramatic reduction of 84.5% with siRNA-2, 63.1% with siRNA-3, and 80.7% with siRNA-4 in the levels of LASS2/TMSG1 mRNA after transfection of siRNA in PC-3M-2B4 cells, compared with LASS2/TMSG1 AllStar Negative Control siRNA or the untransfected cultures ($P < 0.05$; Fig. 1).

Furthermore, the expression of LASS2/TMSG1 protein was detected by Western blot before and after interference, which revealed that LASS2/TMSG1 siRNA-2 suppressed LASS2/TMSG1 production to about 40% of that in the control cultures as shown in Figure 2 ($P < 0.05$).

Therefore, this study selected siRNA-2 as an effective siRNA for subsequent experiments.

siRNA AGAINST LASS2/TMSG1 HAS NO EFFECT ON THE EXPRESSION AND SECRETION OF MMP-2 PROTEIN

In total protein of the PC-3M-2B4 cells and cell supernatant, there was no significant difference in the expression of MMP-2 protein among LASS2/TMSG1 siRNA-2, AllStar Negative Control siRNA or the untransfected cultures (Fig. 3), indicating that the down-regulation of LASS2/TMSG1 expression did not affect the expression and secretion of MMP-2 protein.

siRNA AGAINST LASS2/TMSG1 INCREASES THE ACTIVITY OF MMP-2 IN THE SUPERNATANT OF THE CULTURED CELLS

The activity of MMP-2 in LASS2/TMSG1 siRNA-2 transfected group was approximately fourfold higher than that in the untransfected group and Allstar Negative Control siRNA group ($P < 0.05$; Fig. 4).

siRNA AGAINST LASS2/TMSG1 INCREASES 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/

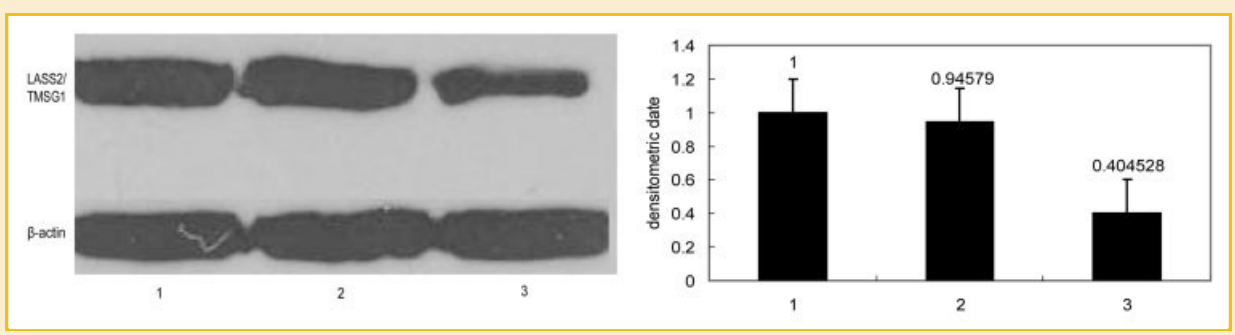

Fig. 2. A: The expression of LASS2/TMSG1 protein was detected before and after interference. B: Western blot revealed that LASS2/TMSG1 siRNA-2 suppressed LASS2/TMSG1 production to about 40% of that in the control cultures ($P < 0.05$). 1: the untransfected group, 2: AllStar Negative Control siRNA group, 3: siRNA-2 targeting LASS2/TMSG1 group.

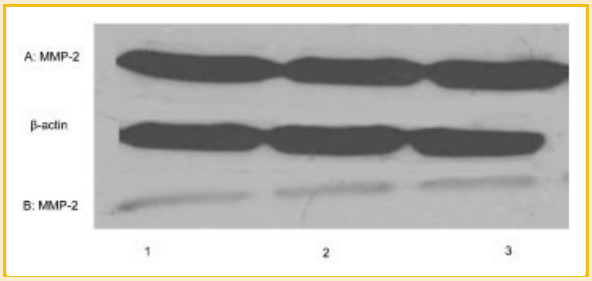

Fig. 3. The matrix metalloproteinase-2(MMP-2) protein in the PC-3M-2B4 cells (A) and supernatant (B) was analyzed by Western blot. There was no significant difference in the expression and the secretion of MMP-2 protein among LASS2/TMSG1 siRNA-2, LASS2/TMSG1 AllStar Negative Control siRNA or the untransfected cultures. 1: The untransfected group, 2: AllStar Negative Control siRNA group, 3: siRNA-2 targeting LASS2/TMSG1 group.

(concentration $\times$ 31.1) $\times$ 10^3 U/mg. The results showed that the V-ATPase activity in LASS2/TMSG1 siRNA-2 treated 2B4 cells (2.286 ± 0.35) increased with statistical significance compared with untreated 2B4 cells (1.142 ± 0.43) or Allstar Negative Control siRNA treated 2B4 cells (1.327 ± 0.202; $P < 0.05$, $n = 3$), as shown in Table I.

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

Extracellular H^+ concentration was detected by pH-sensitive fluorescence probe bis-carboxyethyl-carboxyfluorescein (BCECF), as shown in Figure 5. The proton secretion of LASS2/TMSG1 siRNA-2 treated cells was notably increased at 12 and 24 h compared with that of untreated cells or Allstar Negative Control siRNA treated cells ($P < 0.05$, $n = 3$). No difference was observed between the untreated cells and Allstar Negative Control siRNA treated cells.

siRNA TARGETING LASS2/TMSG1 PROMOTES THE MIGRATION AND INVASION OF PC-3M-2B4 CELLS

In wound migration assay, the cell-free wound gaps of PC-3M-2B4 cells monolayers healed slowly in the untreated cells or Allstar Negative Control siRNA transfected cells. However, in LASS2/TMSG1 siRNA-2 transfected cells, the closure of the wounded area was significantly accelerated (Fig. 6A). The cell numbers of migration in the untreated cells, Allstar Negative Control siRNA transfected cells and the siRNA-2 transfected cells at 24 h after wounding were: 30 ± 2.12, 31 ± 1.99, and 90 ± 2.5, respectively, the difference was statistically significant ($P < 0.01$).

Furthermore, using Boyden chamber invasion assay, the LASS2/TMSG1 siRNA-2 transfected cells displayed dramatically increased invasion ability when compared with the untreated cells or Allstar Negative Control siRNA transfected cells (Fig. 6B). The cell numbers of invasion in the untreated group, Allstar Negative Control siRNA transfected group and LASS2/TMSG1 siRNA-2 transfected group were: 11 ± 1.974, 10.07 ± 1.39, and 21.2 ± 1.21, respectively. The difference was statistically significant ($P < 0.05$).

Therefore, siRNA-2 targeting LASS2/TMSG1 significantly increased the migration and invasion ability of PC-3M-2B4 cells.

DISCUSSION

In 1988, the first metastasis-suppressor gene (MSG), nm23, was cloned from non-metastatic and metastatic variants K-1735 cells of malignant melanoma using differential gene expression techniques by Steeg et al. [1988]. From then on, some metastasis associated gene were cloned and identified in succession. In 2001, Erich B. Jaeger advanced the definition of metastasis suppressor genes: metastasis suppressor genes (MSGs) are defined as genes that do not affect cell growth of primary tumor cells, but can inhibit development of distant metastasis [Jaeger et al., 2001]. Among these MSGs, LASS2/TMSG1 is discovered as a novel tumor metastasis suppressor gene in 1999. Our study found that the expression of LASS2/TMSG1 protein was high in non-metastatic human prostate cancer cell line PC-3M-2B4 [Ma et al., 2002] and human pulmonary giant cell carcinoma cell line PG-LH7 [Bian et al., 2003], but low in the isogenetic metastatic variants PC-3M-1E8 and PG-BE1 cells. Using sense and antisense cDNA transfection, LASS2/TMSG1 was found to significantly suppress the invasion ability of human prostate cancer cell line PC-3M, human pulmonary giant cell carcinoma cell line PG, and human breast cancer cell line MDA-MB-231 [Bian et al., 2003; Su et al., 2007, 2008]. Further study on LASS2/TMSG1 confirmed the following by our laboratory and other study groups. (1) LASS2/TMSG1 gene was located on chromosome 1q21.3; (2) Western blot and immunohistochemistry showed LASS2/

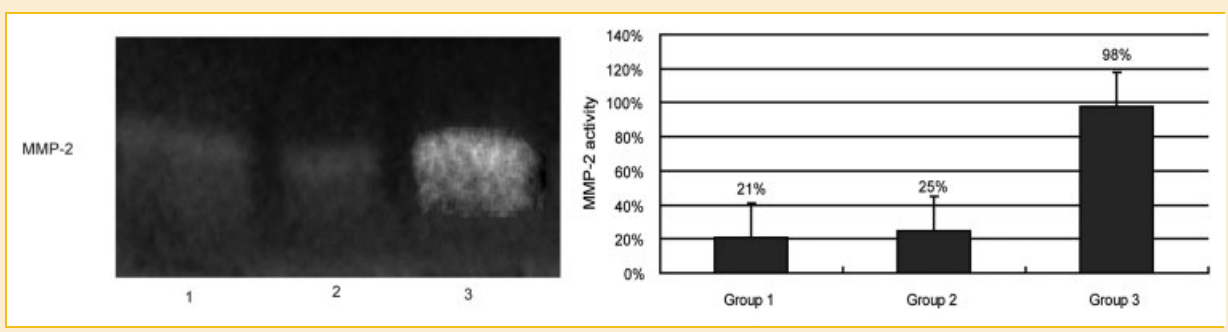

Fig. 4. The activity of MMP-2 was examined by Gelatin zymography. The activity of MMP-2 in LASS2/TMSG1 siRNA-2 transfected group was approximately fourfold higher than that in the untransfected group and Allstar Negative Control siRNA group ($P < 0.05$). 1: The untransfected group, 2: AllStar Negative Control siRNA group, 3: siRNA-2 targeting LASS2/TMSG1 group.

TABLE I. V-ATPase Activity of Three Groups

Cell lines	V-ATPase activity			
	First	Second	Third	$\overline{X} \pm SD$
The untreated 2B4 cells	1.374	1.0491	1.005	1.143 ± 0.202
Allstar Negative Control siRNA treated 2B4 cells	1.823	1.088	1.0686	1.327 ± 0.43
LASS2/TMSG1 siRNA treated 2B4 cells[a]	2.691	2.088	2.08	2.286 ± 0.35

[a]The V-ATPase activity in LASS2/TMSG1 siRNA treated 2B4 cells increased with statistical significance compared with untreated 2B4 cells or Allstar Negative Control siRNA treated 2B4 cells ($P < 0.05$, n = 3).

TMSG1 protein was excellent inverse correlation to tumor metastasis potential, with the molecular weight of 45 kDa (supporting the encoded protein containing 380 amino acids) and localization in cytomembrane and cytoplasm of tumor cell [Fei et al., 2004]; (3) 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 (ASPRH1) and type 2 (ASPRH2), the organic cation transporter-1 (OCT1) and the proteolipid subunit of vacuolar H^+- ATPase (VPL), which suggested LASS2/TMSG1 was a membrane-association protein [Pan et al., 2002]. It was notable that LASS2/TMSG1 associated with subunit C of V-ATPases.

Asialoglycoproteins receptor exists only in the surface of hepatic cell, and there is not asialoglycoproteins receptor in prostate cells [Eisenberg et al., 1991]. Organic cation transporter-1(OCT1) [Shikata et al., 2007] mainly mediated the facilitated transport of a variety of structurally diverse organic cations, including many drugs, toxins, and endogenous compounds. At present, we have not seen the study about OCT1 and tumor. And many literatures [Goldstein et al., 1992; Skinner and Wildeman, 1999; Liotta and Kohn, 2001; Cardone et al., 2005] reporeted that Vacuolar H^+-ATPase can participate in the invasion and metastasis of carcinoma cells. Therefore, we singled out vacuolar H^+-ATPase for this study.

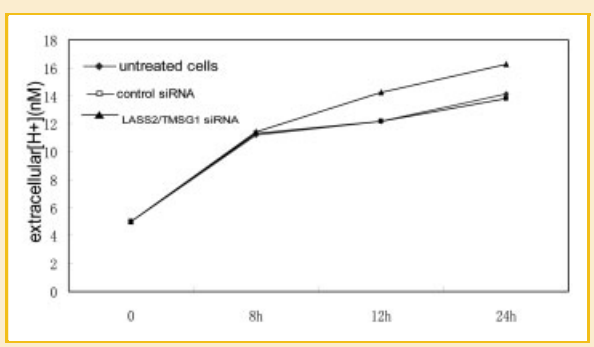

Fig. 5. The concentration of extracellular hydrogen ion was measured by pH-sensitive BCECF. The proton secretion of LASS2/TMSG1 siRNA-2 treated cells was notably increased at 12 and 24 h compared with that of the untreated cells or Allstar Negative Control siRNA treated cells ($P < 0.05$, n = 3). No difference was observed between the untreated cells and Allstar Negative Control siRNA treated cells.

However, as a novel tumor metastasis suppressor gene, the precise molecular mechanisms of LASS2/TMSG1 on cancer metastasis through V-ATPase is unclear. Therefore, to further investigate the molecular mechanisms of LASS2/TMSG1's anti-tumor effect on metastasis of prostate cancer, small interfering RNAs (siRNA) targeting LASS2/TMSG1 was adopted to silence the gene expression in human prostate cancer cell line PC-3M-2B4 (low metastatic potential, high expression of LASS2/TMSG1).

Above all, our results showed that siRNA targeting LASS2/TMSG1 could promote the migration and invasion of PC-3M-2B4 cells, which in turn suggested that LASS2/TMSG1 gene had the function of tumor metastasis suppressor. 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 [Ning et al., 2007]. Besides, they also discovered that small interfering RNA targeting LASS2/TMSG1 gene could enhance invasion capacity of human hepatocellular cell line MHCC97-L [Ning et al., 2009]. The researches above suggested that LASS2/TMSG1 was a novel tumor metastasis suppressor gene.

It is well-known that extracellular pH is usually low in solid tumors, in contrast to the approximately neutral intracellular pH. V-ATPase, which overly functions in some cancers with metastatic potential, plays an important role in maintaining neutral cytosolic pH, very acidic luminal pH, and acidic extracellular pH. The subunit C of V-ATPases (ATP6L), the 16 kDa subunit of proton pump V-ATPase, can provide proton hydrophilic transmembrane path. Interestingly, our results detected that siRNA against LASS2/TMSG1 could enhance the activity of V-ATPase in PC-3M-2B4 cells by GEMEND's V-ATPase activity assay kit and increase extracellular H^+ concentration in PC-3M-2B4 cells using pH-sensitive BCECF. The findings were also confirmed by Shanghai research group. They found that LASS2/TMSG1 siRNA could increase the proton transmembrane secretion in MHCC97-L cells by measuring extracellular pH with pH-sensitive BCECF [Ning et al., 2009]. These results suggested that LASS2/TMSG1 could inhibit the activity of V-ATPase, suppress the proton transmembrane secretion and thus decrease extracellular H^+ concentration in cancer cells. The vacuolar (H^+)-ATPases (or V-ATPases) are a family of ATP-dependent proton pumps that are responsible for acidification of intracellular compartments in eukaryotic cells and were found on the membranes of lysosomes, the Golgi complex, secretory granules, and some specific region of cytomembrane [Forgac, 1999]. In addition to their role in intracellular compartments, V-ATPases have also been shown to play an important role in the plasma membrane

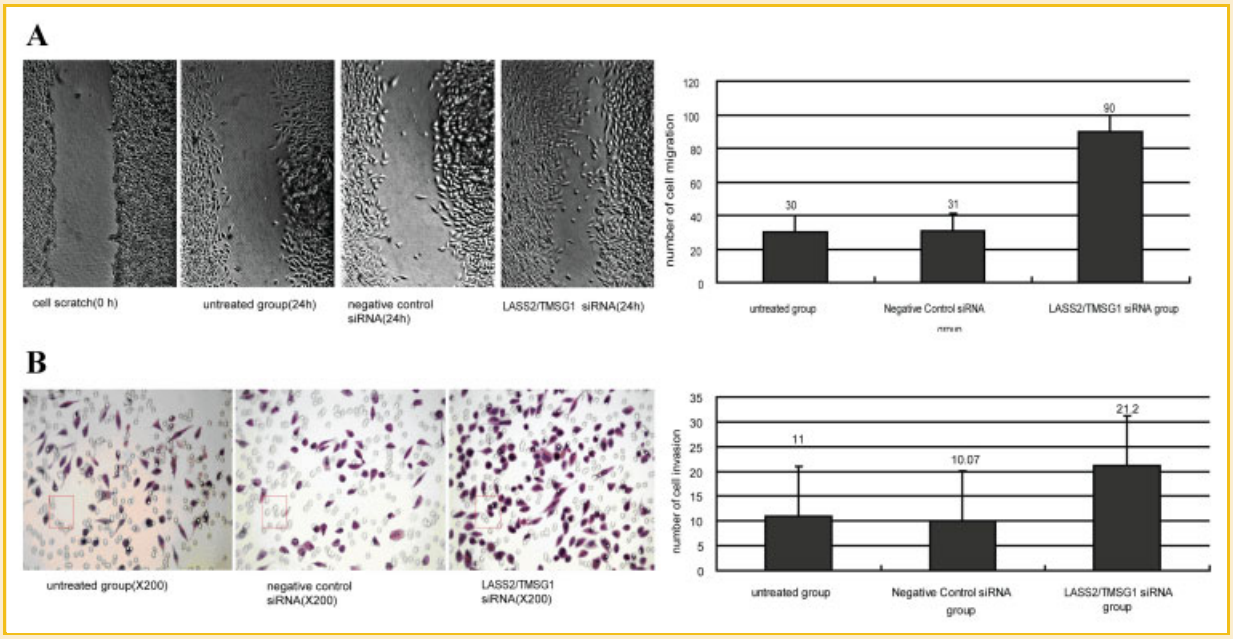

Fig. 6. siRNA targeting LASS2/TMSG1 promotes the migration and invasion of PC-3M-2B4 cells. A: In wound migration assay, the cell-free wound gaps of PC-3M-2B4 cell monolayers healed slowly in the untreated cells or Allstar Negative Control siRNA transfected cells. However, in LASS2/TMSG1 siRNA transfected cells, the closure of the wounded area was significantly accelerated ($P < 0.01$, $n = 3$); B: Using Boyden chamber invasion assay, the LASS2/TMSG1 siRNA transfected cells displayed dramatically increased invasion ability when compared with the untreated cells or Allstar Negative Control siRNA transfected cells ($P < 0.05$, $n = 3$). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

of various specialized cells, such as renal cells, osteoclasts, and tumor cells. Tumor cells are able to target V-ATPases to the plasma membrane where they create an acidic extracellular environment that is necessary for tumor metastasis. The promoting effect of V-ATPase on cancer invasion and metastasis mainly relies on its maintaining acidic pH of extracellular microenvironment, which is related to the activation, secretion, and cellular distribution of many proteases involved in the digestion of ECM [Sennoune et al., 2004b]. The pH-sensitive proteases include cathepsin and MMPs (MMP-2, MMP-9, MMP-3, etc.). Besides, another mechanism that the active V-ATPase could facilitate invasion and metastasis may be through its influence on cellular motility and migration. In the activated ostoeblasts, both the V1 complex and the holoenzyme of V-ATPase can interact with the actin-based cytoskeleton, contributing to the densely packed V-ATPase at the ruffled border adjacent to the sealed compartment where bone matrix is absorbed [Lee et al., 1999]. From the viewpoint that the invasive phenotype of malignant cells is somehow similar to the activated ostoeblasts, the concentration of V-ATPase may probably occur in the cell surface of an invading cancer cell, resulting in the "aggressive" protruding edge [Chen et al., 2004]. In summary, siRNA against LASS2/TMSG1 in PC-3M-2B4 cells enhanced the activity of V-ATPase, accelerated the proton transmembrane secretion and thus increased extracellular H^+ concentration, which activated many proteases involved in the digestion of ECM, enhanced cellular motility and migration and eventually led to promote the migration and invasion of cancer cells.

Finally, we discovered that siRNA against LASS2/TMSG1 could increase the activity of MMP-2 in the supernatant of the PC-3M-2B4 cells, but had no effect on the expression and secretion of MMP-2 protein. The results were similar to Shanghai research group. They found that LASS2/TMSG1 siRNA could up-regulate the activation of MMP-2 in MHCC97-L cells [Ning et al., 2009]. Some results indicated that migration of tumor cells is regulated by MMP activity, rather than by MMP expression. Furthermore, there is no linear relationship between MMP2 expression following transfection and the ability of cancer-cell lines to invade in a matrigel assay [Egeblad and Werb, 2002].

In conclusion, silencing of LASS2/TMSG1 can promote invasion of prostate cancer cell in vitro through the increase of the V-ATPase activity and extracellular hydrogen ion concentration, and in turn the activation of secreted MMP-2 and degradation of ECM, which ultimately accelerated tumor's invasion and metastasis, indicating that LASS2/TMSG1 is a novel tumor metastasis suppressor gene.

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