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A Novel Tumor Metastasis Suppressor Gene LASS2/TMSG1 Interacts with Vacuolar ATPase Through Its Homeodomain

Wenjuan Yu,[1,2] Leiming Wang,[1] Yuewei Wang,[3] Xiaoyan Xu,[1] Pengcheng Zou,[1] Miaozi Gong,[1] Jie Zheng,[1] Jiangfeng You,[1] Hua Wang,[1] Fang Mei,[1] and Fei Pei[1*]

[1] *Department of Pathology, School of Basic Medical Sciences, Peking University Health Science Center, Beijing 100191, P.R. China*

[2] *Department of Pathology, The Affiliated Hospital of Medical College, Qingdao University, Qingdao 266003, P.R. China*

[3] *Department of Vascular Surgery, The Affiliated Hospital of Medical College, Qingdao University, Qingdao 266003, P.R. China*

ABSTRACT

LASS2/TMSG1 was a novel tumor metastasis suppressor gene, which was first cloned by our laboratory from non-metastatic and metastatic cancer cell variants of human prostate carcinoma PC-3M using mRNA differential display in 1999. LASS2/TMSG1 could interact with the C subunit of vacuolar ATPase (V-ATPase, ATP6V0C) and regulate V-ATPase activity. In an attempt to provide molecular mechanism of the interaction between LASS2/TMSG1 and V-ATPase, we constructed four variant transfectants containing different functional domain of LASS2/TMSG1 and stably transfected the variants to human prostate cancer cell line PC-3M-1E8 cell with high metastatic potential. Results showed that there were no obvious differences of V-ATPase expression among different transfected cells and the control. However, V-ATPase activity and intracellular pH was significantly higher in the variant transfectants with Homeodomain of LASS2/TMSG1 than that in the control using the pH-dependent fluorescence probe BECEF/AM. Immunoprecipitation, immunofluorescence and immuno-electron microscope alone or in combination demonstrated the direct interaction of Homeodomain of LASS2/TMSG1 and ATP6V0C. Loss of Homeodomain markedly enhanced the proliferation ability but weakened the apoptotic effect of LASS2/TMSG1 in PC-3M-1E8 cells. These lines of results for the first time contribute to the conclusion 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. Their interaction could play important roles in the apoptosis of tumor cells. J. Cell. Biochem. 114: 570–583, 2013.

KEY WORDS: TUMOR METASTASIS SUPPRESSOR GENE; LASS2/TMSG1; PROSTATE CANCER; VACUOLAR ATPASE; HOMEODOMAIN

LASS2/TMSG1 gene (tumor metastasis suppressor gene 1) is a novel tumor metastasis suppressor gene, which was first cloned by our laboratory from non-metastatic and metastatic cancer cell variants of human prostate carcinoma using mRNA differential display in 1999 [Ma et al., 2002] (GenBank Accession No. AF189062 [Strausberg et al., 2002]). Furthermore, the identification of the gene was confirmed successively by other laboratories. In September 2001, LASS2 (Homo sapiens longevity assurance homologue 2 of yeast LAG1) (GenBank 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; Laviad et al., 2008]. LASS2 could bind to the asialoglycoprotein receptor, organic cation transporter-1 and the proteolipid subunit of vacuolar H^+-ATPase in vitro. In April 2008, Laviad found there were two alternatively spliced transcripts of LASS2/TMSG1 gene encoding the same protein [Forgac, 1998]. 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 acyl chain ceramides.

Meanwhile, it was found that protein encoded by LASS2/TMSG-1 could interact with the C subunit of V-ATPase [Pan et al., 2001], which suggested that LASS2/TMSG1 might inhibit the invasion and

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*Correspondence to: Fei Pei, MD, PhD, Research and Clinical Associate Professor, 38 Xue Yuan Road, Haidian District, Beijing 100191, P.R. China. E-mail: peifei@bjmu.edu.cn

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metastasis of tumor cells through regulating the function of V-ATPase. V-ATPase (Vacuolar-Type [H^+]-ATPases) is a ubiquitous proton-translocating pump 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. The C subunit of V-ATPase (ATP6V0C) plays an important role in the construction of protons channel, through which protons are pumped out of the cytoplasm into the organelle or the extracellular space, which mainly assumes the responsibility of regulating the cytoplasmic pH of mammalian cells. V-ATPase-mediated regulation of intracellular pH (pHi) contributes to maintain intracellular pHi within proper values that are permissive for cell growth and function, in addition to regulating cancer drug resistance and cell apoptosis [Gillies and Martinez-Zaguilan, 1992; Forgac, 1998]. Moreover, V-ATPase is expressed in plasma membrane of some tumor cells, which is correlated with the metastatic potential of these cells. However, the molecular mechanism by which LASS2/TMSG1 on cancer metastasis through V-ATPase is unclear at present.

The coded protein sequence of LASS2/TMSG1 comprised two conserved functional domains: Homeodomain (67–128aa) and TRAM-LAG1-CLNS (TLC) domain (131–332aa). Homeodomain proteins are a series of recognizable proteins coded by homeobox genes, which were characterized by the possession of a particular DNA sequence. A majority of Homeodomain proteins are regarded as transcription factors with important roles in the cellular growth, differentiation and embryonic development [McGinnis and Krumlauf, 1992; Myers et al., 2000]. It was noticeably, increasing studies manifested the considerable relationship between Homeodomain and tumor, such as CDX2, which was found to possess significant tumor inhibitory functions [Borrow et al., 1996; Brabletz et al., 2004; Gross et al., 2008]. Furthermore, TLC domain was a characteristic domain of LASS family with high conservatism, which was important to the synthesis of ceramide, especially the long acyl-chain C20–C26 fatty acids. It was now well established that ceramide was a kind of tumor-suppressor lipid involved in promoting cell apoptosis and cell cycles arrest [Lee et al., 2000; Ogretmen et al., 2001; Ogretmen and Hannun, 2004].

Based on the previous conclusions that LASS2/TMSG1 could interact with the C subunit of V-ATPase, and the Homeodomain and TLC domain both could play tumor-suppressor function, there is a particular concern about which domain of LASS2/TMSG1 was the key point bearing the interaction of LASS2/TMSG1 and ATP6V0C. As far as we know, there was no comprehensive survey about this up till now.

In an attempt to give an insight into the mechanism through which LASS2/TMSG1 impacts V-ATPase activity, and find the functional domain of LASS2/TMSG1 interacting with ATP6V0C, we constructed four eukaryotic expression vectors containing different domains of LASS2/TMSG1 and transfected the vectors to human prostate carcinoma cell line PC-3M-1E8 cell with high metastatic potential and detected the expression of V-ATPase in mRNA and protein levels. Then we detected the activity of V-ATPase in addition to the pHi. Sensitive methods containing immunoprecipitation, immunofluorescence, and immuno-electron microscope were applied to intuitively identify the direct interactive domain of LASS2/TMSG1 with ATP6V0C.

MATERIALS AND METHODS

CELL CULTURE

Human prostate cancer cell lines PC-3M-1E8 cells (high-metastatic cancer variant from human prostate cancer cell PC-3M; tumorigenicity frequency in nude mice: 100%; spontaneous metastasis frequency in nude mice: 100%) were established by Molecular Pathology Laboratory, Department of Pathology, Peking University Health Science Centre [Liu et al., 1999]. PC-3M-1E8 and PC-3M-2B4 (low-metastatic cell variant from human prostate cancer cell PC-3M) cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. HEK293T cells were cultured in DMEM medium supplemented with 10% fetal calf serum.

CONSTRUCTION OF VECTOR, GENE TRANSFECTIONS AND CLONES SELECTION

The variants, containing TLC domain (T1) (nucleotides 778–1,530 of the human LASS2/TMSG1 cDNA), Homeodomain and TLC domain (T2) (nucleotides 571–1,530 of the human LASS2/TMSG1 cDNA), the first transmembrane region and Homeodomain (T3) (nucleotides 388–771 of the human LASS2/TMSG1 cDNA), and the full-length coding region of human LASS2/TMSG1 gene (T4) was amplified from PC-3M-2B4 cDNA library using different special primers, respectively (Fig. 1, Table I). The PCR products were cloned into the enzymatic site between *Eco*RI and *Xho*I of the eukaryotic expression vector pcDNA3 (Invitrogen, Carlsbad, CA, USA) with FLAG tag in the carboxyl terminus. Therefore, four vectors were established called T1, T2, T3, and T4, which contained the different variants of LASS2/TMSG1 gene as described above. After correct sequencing, the recombined plasmid was extracted by Pureyield^{TM} plasmid Midprep extraction kit (Promega, Madison, WI, USA). Stable transfection of PC-3M-1E8 cells was performed according to the manufacturer's instruction for Lipofectamine^{TM} 2000 (Invitrogen). After 48–72 h, positive clones for subsequent studies were preliminary isolated by maintaining the cells in the medium containing 600 μg/ml G418 (Gibco BRL, Gaithersburg, MD, USA). Ultimately, two aimed clones of every variant were identified by real-time RT-PCR (QPCR) and Western blot analysis, which presented high expressions of the variants from the positive clones. (Primers were shown in Table I.) The primary antibody was anti-FLAG (1:500, Sigma, St Louis, MO, USA). The PC-3M-1E8 transfected by pcDNA3 and the untransfected parental PC-3M-1E8 cells were regarded as the controls.

GENE EXPRESSION ANALYSIS OF ATP6V0C BY QPCR

The mRNA levels of ATP6V0C in PC-3M-1E8 and PC-3M-1E8 with different variants transfection were detected by ABI 7500 Fast Real-time Polymerase Chain Reaction System (Applied Biosystems, Inc., Carlsbad, CA, USA). Total RNA and cDNA of PC-3M-1E8 and PC-3M-2B4 cells were prepared by Trizol reagent (Invitrogen) and M-MLV reverse transcriptase (Progema). The primers specifically for cDNA rather than genome DNA were designed by primer premier 5.0 (Table I). The parameters for PCR were: 95°C, 3 min; 95°C, 30 s; 60°C, 30 s; 72°C 30 s, 40 cycles.

PROTEIN EXPRESSION OF ATP6V0C BY WESTERN BLOT

The protein levels of ATP6V0C in PC-3M-1E8 with different variants transfection were detected by Western blot. The total protein was

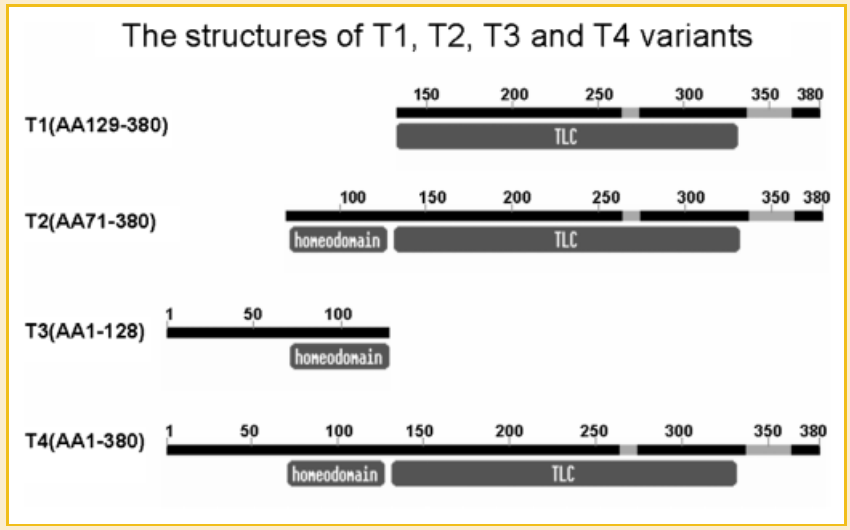


Fig. 1. The schematic diagram showing the domains of T1, T2, T3, and T4 variants.

extracted by 2× SDS cell lysis solution (100 mM Tris–HCl pH 6.8, 4% SDS, 20% glycerol, 200 mM DTT, 0.2% bromophenol blue). Proteins were separated by 12% SDS–PAGE gels and transferred to 0.22 μm nylon membrane. The following antibodies were applied: anti-ATP6V0C (1:500, Abnova, Taiwan, China), anti-Tubulin (1:400, Neomarker, Fremont, CA, USA). ECL chemoluminescence reagent was obtained from Pierce Biotechnology.

V-ATPASE ACTIVITY ANALYSIS

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, and 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 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, and 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 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/l EDTA, 3 mmol/l ATP, 0.1 mmol/l ammonium molybdate, 5 μmol/l valinomycin, 5 μmol/l Nigericin, 5 μg/ml Oligomycin, and 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., Wilmington, DE, USA). Absorbency (340 nm) of samples at the 0 min, the 5th min, and the 10th min after beginning of the reaction was measured in reaction medium of malachite green to determine the V-ATPase activity.

INTRACELLULAR PH DETECTION

High potassium HEPES buffer (25 mmol/l HEPES, 5 mmol/l NaCl, 140 mmol/l KCl, 1.8 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, 5.5 mmol/l glucose, and 5 μmol/l Nigericin) at pH 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0 was prepared in order to prepare pHi calibration. Cultured cells in 96-well plates (Corning, Inc., Corning, NY, USA) were washed with the high potassium HEPES buffer of different pHi mentioned above, each twice for 5 min. Cells were incubated in the solutions with different pHi containing 3 μmol/l BCECF/AM (Invitrogen) for 30 min at 37°C in 5% CO_2. When the incubating solution was

TABLE I. Sequences of Oligonucleotides Used as Primers for Plasmid Constructs and Real-Time PCR

Primers	Sequences
Plasmid construct	
T1	
Sense	5′-CGCGAATTCGCCGCCACCATGCTCCTCAAGAAGTTCCGAG-3′
Antisense	5′ -CGCCTCGAGGTCATTCTTACGATGGTTGTTATT-3′
T2	
Sense	5′-CGCGAATTCGCCGCCACCATGACACCACTGGCTGCCCTCTTG-3′
Antisense	5′-CGCCTCGAGGTCATTCTTACGATGGTTGTTATT-3′
T3	
Sense	5′-CGCGAATTCGCCGCCACCATGCTCCAGACCTTGTAT-3′
Antisense	5′-CGCCTCGAGGAGGAGACTGGGCCGGT-3′
T4	
Sense	5′-CGCGAATTCGCCGCCACCATGCTCCAGACCTTGTATGATTA-3′
Antisense	5′-CGCCTCGAGGTCATTCTTACGATGGTTGTTATT-3′
Real-time PCR	
ATP6V0C	
Sense	5′-CCCCGA GTATGCTTCGTTTT-3′
Antisense	5′-TGATGCCAGCC ATGACCACT-3′
T1	
Sense	5′-AAGAAAGTTTGGGAGGGA-3′
Antisense	5′-CCAGCAGGTAATCGGAAG-3′
T2	
Sense	5′-ATACAGAGCACTATCCCTTCC-3′
Antisense	5′-CAGGTGTTCTTCCATCCC-3′
T3	
Sense	5′-TCTACGCCAAAGCCTCAG-3′
Antisense	5′-CATCATCGTCCTTATAGTCCC-3′
T4	
Sense	5′-CTGCTGCATATCTTCTGG-3′
Antisense	5′-TTTGTCATCATCGTCCTT-3′

discarded, the cells were washed twice with PBS of different pHi, and then measured in a 96-well fluorospectrophotometer. The standard curve was made according to the value of A540/A620 (FIR) indicated by the fluorospectrophotometer, which represented the fluorescence density of the corresponding pHi. The experimental cells were washed with HEPES buffer (25 mmol/l HEPES, 5 mmol/l NaCl, 140 mmol/l KCl, 1.8 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, and 5.5 mmol/l glucose) at pH 7.4 twice for 5 min. Cells were incubated in the same solution containing 3 μmol/l BCECF/AM for 30 min at 37°C in 5% CO_2. When the incubating solution was discarded, the cells were washed twice with PBS of pH 7.4, and then measured in a 96-well fluorospectrophotometer. The pHi was calculated according to the density of fluorescence through the aforementioned standard curve. Aside from the detection by fluorospectrophotometer, the fluorescence of BCECF/AM was observed under the laser passing confocal microscopy and the fluorescence density of different groups was calculated under the same parameters.

CONSTRUCTION, TRANSFECTION AND IDENTIFICATION OF SIRNAS TARGETING HUMAN ATP6V0C

We designed and purchased four different siRNA duplexes of ATP6V0C (GenBank Accession No. NM001694) from Qiagen Company (Dusseldorf, Germany). The targeting sequence of four siRNA were as follows: SIRNA1: CACAAAGTAGACCCTCTCCGA; SIRNA2: CCCAC-CAGCCACA GAATATTA; SIRNA3: TGCGCGGAGCTGTGTCCAATA; and SIRNA4: GCGGATGATTTAGAATTGTCA. The nonspecific control siRNA duplexes (AllStar Negative Control siRNA) were also purchased from Qiagen Company. Transfection of siRNA targeting ATP6V0C and AllStars Negative Control siRNA into PC-3M-1E8 cells was done using Lipofectamine™ 2000. Briefly, 3×10^5 cells were seeded per well into six-well plates. The cells were transfected using 0.1 nmol siRNA-1, siRNA-2, siRNA-3, siRNA-4, and AllStars Negative Control siRNA (20 μmol/l) mixed with 5 μl Lipofectamine™ 2000 in 500 μl medium without serum. After 4–6 h incubation, the culture medium was disposed and new RPMI1640 culture medium containing 10% fetal bovine serum was added to cultivate for 24 h. Native PC-3M-1E8 cells were treated as blank control. Then total RNA and cDNA of PC-3M-1E8 and the control cells were prepared by Trizol reagent (Invitrogen) and M-MLV reverse transcriptase (Progema). The primers specifically for cDNA rather than genome DNA were designed by primer premier 5.0 (Table I). The parameters for PCR were: 95°C, 3 min; 95°C, 30 s, 60°C, 30 s, and 72°C, 30 s, 40 cycles. The experiment was done in triplicates.

TRANSFECTION OF T1, T2, T3, AND T4 TO PC-3M-1E8 CELLS AFTER THEY WERE TRANSFECTED BY THE EFFECTIVE SIRNA OF ATP6V0C, AND THE DETECTION OF PHI

First, six groups of PC-3M-1E8 cells in six-well plates were transiently transfected by the effective siRNA of ATP6V0C. After 24 h, the cells were then transfected by T1, T2, T3, and T4 and pcDNA3, respectively. About 24 h later, the cells were seeded to 96-well plates, cultured for 24 h, and then the pHi was detected by 96-well fluorospectrophotometer after the staining of BCECF/AM. The detailed procedures for transfection and pHi detection were the same as the aforementioned.

IMMUNOPRECIPITATION ASSAY

For immunoprecipitation, the variants and ATP6V0C cotransfected HEK293T cells were solubilized in 500 μl cold NP-40 lysis buffer (1% NP-40, 50 mM Tris-buffer saline pH 8.2, 100 mM NaCl, and 5 mM EDTA) containing 5 μl protease inhibitor cocktail and 5 μl PMSF (100 mM) for 30 min and then centrifuged for 15 min at 4°C, 12,000 rpm. Then 50 μl protein G agarose beads were added into the collected supernatant fluid for ruling out the nonspecific IgG. Then the immunosorbent-rabbit anti-human FLAG antibody (2 μg, Sigma) and 50 μl protein G agarose beads were added in and the mixture was rotated at 4°C, 100 rpm for 12 h. Beads were washed three times with PBS containing protease inhibitor cocktail and PMSF. Then 2× SDS loading buffer (100 mM Tris–HCl pH 6.8, 4% SDS, 20% glycerol, 200 mM DTT, and 0.2% bromophenol blue) was added into the precipitation and boiled for 10 min. After centrifuge, the supernatant was collected for further Western blot analysis using mouse antihuman ATP6V0C antibody (1:250) purchased from Abnova, and mouse antihuman FLAG antibody (1:500) purchased from Sigma.

INDIRECT IMMUNOFLUORESCENCE BY DOUBLE STAINING AND FRET ANALYSIS

The variants stable-transfected PC-3M-1E8 cells were seeded on slides in RPMI 1640 containing 10% FBS. About 24 h after attachment, the cells were fixed in acetone for 30 min and then rinsed with PBS twice. The cells were treated with 1% BSA: PBS for 20 min at room temperature for the blockage of nonspecific antigen, and incubated with the rabbit polyclonal antibody against FLAG (1:500) and mouse monoclonal antibody against ATP6V0C (1:250) for 2 h at 37°C. After rinse with PBS twice for 5 min, Alex fluorescence 488-conjugated goat anti-rabbit IgG (1:100 dilution, Invitrogen) and Cy3-conjugated goat anti-mouse IgG (1:100 dilution, Invitrogen) were added in and incubated for 30 min at 37°C. The slides were then mounted with mounting medium, and examined by Leica TCS SP5 fluorescence microscope. Fluorescence Resonance Energy Transfer (FRET) is a technique, which allows insight into the interactions between proteins or molecules in proximities beyond light microscopic resolution. Sensitized Emission, one established method for the evaluation of FRET efficiencies was applied. The principle of SE was an excited fluorophore, called the donor, transfers its excited state energy to a light-absorbing molecule, which is called the acceptor. This transfer of energy is nonradiative. FRET sample preparations included references of donor in the absence of the acceptor (donor only control) and acceptor in the absence of the donor (acceptor only control) to obtain calibration coefficients to correct for excitation and emission cross talk. Throughout the entire experiment and calibration routine, all measuring parameters such as gain, emission detection window, excitation intensities, zoom, format, scan speed, and pinhole remained constant. The FRET efficiency, which represented the interacting possibility of ATP6V0C and FLAG fusion protein, was calculated by LAS AF BETA VERSION according to the manufacture's instruction.

ELECTRON MICROSCOPE IMMUNOCYTOCHEMISTRY ANALYSIS

The variants stable-transfected PC-3M-1E8 cells were collected, rinsed with PBS and centrifuged at 6,000*g* for 10 min to be a gray

pellet. The pellet was fixed in 1.5% glutaraldehyde for 2 h and then in osmium tetroxide for 20 min. The pellets were embedded in the Lowicryl K4M resin and then semithin sections of 1 μm thick were first made to observe the morphology under the light microscope. After identifying the aimed area, the ultrathin sections were spreaded on the formvar-carbon-coated electron microscope nickel grids for further immunocytochemistry analysis. After blocking by 5% BSA:PBS, the primary rabbit antibody against FLAG (1:500, Sigma) together with mouse antibody against ATP6V0C (1:250, Abnova) were added simultaneously and incubated for 1 h at room temperature. Gold colloid-conjugated goat anti-rabbit IgG (10 nm, 1:20 dilution, Boster technology, Beijing, China) and gold colloid-conjugated goat anti-mouse IgG (15 nm, 1:20 dilution, Boster technology) were immunoreacted with the primary antibody for 45 min at 37°C. The negative controls were as follow: the reaction of second antibody without primary antibody; the primary antibodies replaced by normal rabbit and mouse IgG in parental PC-3M-1E8 cells.

CELL PROLIFERATION ASSAY

MTT assay was performed to evaluate cell proliferation as previously described [Huang et al., 2004]. Briefly, the variants stable-transfected PC-3M-1E8 cells and the controls of the same numbers were seeded in 96-well-plate at 1.0×10^3 cells/well in triplicate after counted. About 20 μl MTT solution (5 mg/ml, Sigma) was added into each well and 150 μl DMSO was added 4 h later. Then the plate was measured for the absorbance at 570 nm by ELISA reader.

FLOW CYTOMETRY FOR THE ANALYSIS OF APOPTOSIS AND CELL CYCLE PROGRESSION

The Apoptosis Assay kit (Gene Research Center of Peking University, Beijing, China) was used to evaluate cell apoptosis. The variants stable-transfected PC-3M-1E8 cells and the controls were digested by 10% trypsin and washed twice by cold PBS; 300-mesh-nylon net was used to filter the cells. Cells were resuspended in 200 μl binding buffer. Then 10 μl Annexin-V-FITC was added and incubated at 4°C for 30 min. Cells were stained with 5 μl propidium iodide (PI) and subjected to flow cytometry analysis.

For the analysis of cell cycle progression, cells were digested by 10% trypsin and washed twice by cold PBS and subsequently fixed by 75% alcohol at 4°C for 24 h; 300-mesh-nylon net was applied to filter the cells and then 10 μl RNAse was added and incubated at 37°C for 30 min. Following staining with PI, samples were subjected to flow cytometry analysis (BD FACSCalibur, Franklin Lakes, NJ, USA).

INVASION ASSAY

The conditional medium of NIH3T3 cells was used as the chemotactic factor in the inferior room of Boyden chamber. A polycarbonate 8-μm thick Millipore membrane was placed between the superior and inferior rooms; 50 μl matrigel (1 μg/L, BD) was evenly distributed on the membrane. After complete polymerization of the matrigel, about 2×10^5 cells were seeded into the superior room of the well. Then the variants stable-transfected PC-3M-1E8 cells and the controls were cultivated at 37°C in 5% CO_2 for 8 h. The cells that crossed the membrane were fixed by methanol and stained by hematoxylin and eosin, and counted under light microscope.

RESULTS

POSITIVE CLONES SELECTION

Amplification of 761 bp (T1), 945 bp (T2), 390 bp (T3), and 1,143 bp (T4) DNA variants could be detected in positive recombinant by RT-PCR using the primers similar to those applied in the vector construction, whereas no DNA amplification was found in the controls (Fig. 2A). Similarly in the positive clones, the proteins expression demonstrated by Western blot parallel with the mRNA expression. The recombined protein mainly migrated to about 25 kDa (T1), 32 kDa (T2), 16 kDa (T3), and 45 kDa (T4) in 10% SDS–PAGE. Two bands were detected in T1, T2, and T4 transfected PC-3M-1E8 cells. It was interesting a band of 20 kDa molecular weight was simultaneously appeared in T1, T2, and T4 transfected PC-3M-1E8 cells (Fig. 2B).

GENE AND PROTEIN EXPRESSIONS OF ATP6V0C PRESENTED NO SIGNIFICANT DISCREPANCY

In order to clarify the influence of over-expression of the Homeodomain and TLC domain of LASS2/TMSG1 on the expression of ATP6V0C, we detected the mRNA expression level of ATP6V0C in the clones with different variants transfection by real-time quantitative RT-PCR (qPCR). Due to the linearity and sensitivity of qPCR, the results demonstrated that ATP6V0C mRNA expression level did not present any variation in different clones and the control cells (Fig. 2C). ATP6V0C protein expression level was in agreement with the mRNA expression level (Fig. 2D).

V-ATPASE ACTIVITY IN T2, T3, AND T4 TRANSFECTED PC-3M-1E8 CELLS PROMINENTLY INCREASED

V-ATPase activity in T1, T2, T3, and T4 transfected PC-3M-1E8 were 0.687 ± 0.114, 2.203 ± 0.284, 1.983 ± 0.176, and 2.373 ± 0.232 U/mg protein, respectively; whereas those in pcDNA3 transfected and parental PC-3M-1E8 were 0.603 ± 0.705 and 0.547 ± 0.114 U/mg protein, which were significantly higher than aforementioned cell line groups excluded T1 transfected cell lines (Fig. 3A).

Intracellular pH in T2, T3, and T4 transfected PC-3M-1E8 was significantly increased compared with the controls.

BCECF/AM is a new kind of pH-dependent fluorescence probe, which is the most widely used fluorescent indicator for pHi because of its high sensitivity and no effect on cell viability. Calibration curve obtained by FIR and corresponding pHi of standard PC-3M-1E8 cells indicated a linear correlation between pHi (6.5, 7.0, 7.5, and 8.0) and FIR detected by fluorospectrophotometer. The obtained regression equation was $Y = 0.207X + 4.994$ (Fig. 3B). According to the FIR, the corresponding pHi of different group was calculated. Results showed FIRs and pHi in T2, T3, and T4 transfected PC-3M-1E8 were significantly increased compared with those in pcDNA3 transfected and parental PC-3M-1E8 as the controls; and there were no differences among T1 and the controls (Fig. 3C). In agreement with the results of fluorospectrophotometer, the laser confocal microscope showed the fluorescence density of BCECF/AM in T1 variant transfected PC-3M-1E8 cell and the controls were

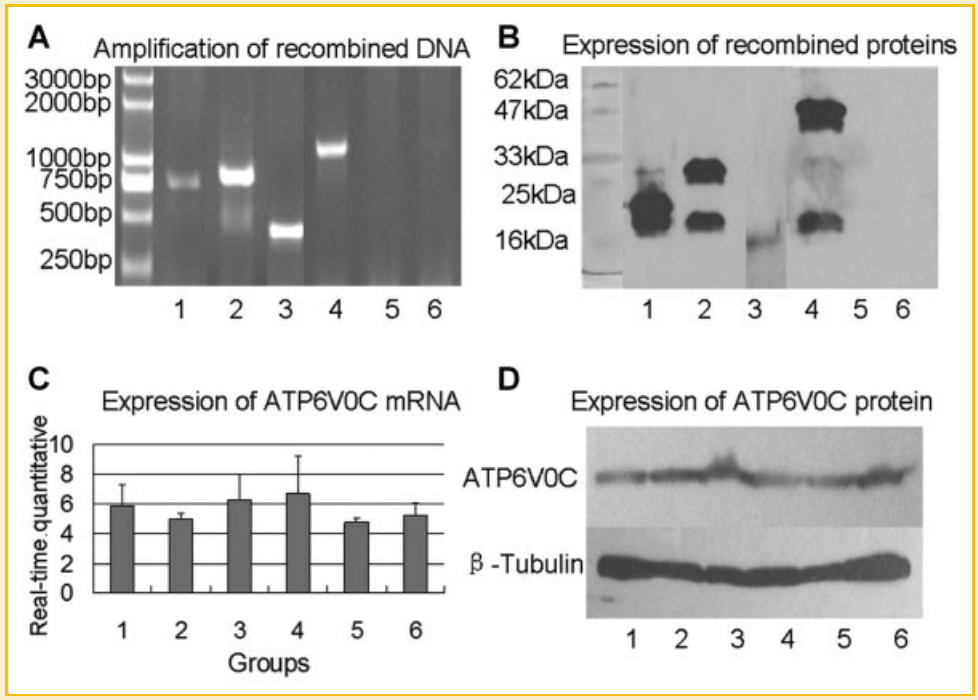


Fig. 2. Expressions of T1, T2, T3, and T4 variants and ATP6V0C. A: Amplification of 761 bp (T1), 945 bp (T2), 390 bp (T3) and 1143 bp (T4) DNA variants could be detected in positive cell clones by RT-PCR. B: The recombined protein in positive clones mainly migrated to about 25 kDa (T1), 32 kDa (T2), 16 kDa (T3) and 45 kDa (T4) in 10% SDS-PAGE, respectively. C: ATP6V0C mRNA expression level did not present any discrepancy in different clones and the controls. D: ATP6V0C protein expression was in agreement with mRNA expression [1, PC-3M-1E8 (T1); 2, PC-3M-1E8 (T2); 3, PC-3M-1E8 (T3); 4, PC-3M-1E8 (T4); 5, PC- 3M-1E8 (pcDNA 3); 6, PC-3M-1E8].

significantly decreased compared with those in T2, T3, and T4 variants transfected cells (Figs. 3D and 4).

EFFECTS OF ATP6V0C SIRNAS ON THE EXPRESSION OF ATP6V0C IN PC-3M-1E8 CELLS

We examined the effects of four siRNAs targeting human ATP6V0C as described above. RT-PCR revealed 71% reduction of ATP6V0C mRNA expression with siRNA-1 transfection, 62% with siRNA-2 transfection, and 87% with siRNA-3 transfection in PC-3M-1E8 cells, compared with AllStar Negative Control siRNA transfected cells and the untransfected cells. The expression of ATP6V0C was not significantly decreased after siRNA-4 transfection compared with the controls (Fig. 5A and B). SiRNA-3 effectively reduced the expression of ATP6V0C mRNA in PC-3M-1E8 cells. Therefore, we selected siRNA-3 as an effective siRNA of ATP6V0C for subsequent experiments.

AFTER THE C SUBUNIT OF V-ATPASE WAS DOWN-REGULATED IN CELLS BY SIRNA, THE INTRACELLULAR PH WAS NOT INCREASED AFTER TRANSFECTION OF T1, T2, T3, AND T4 VARIANTS

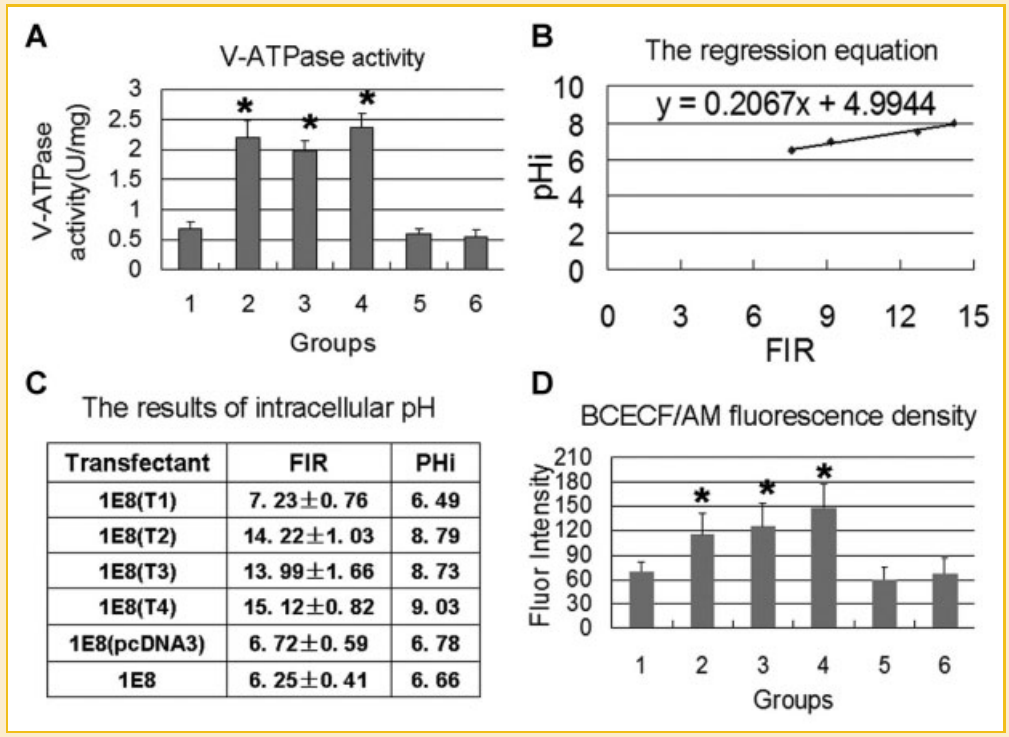
The aforementioned results showed in T2, T3, and T4 variants transfected PC-3M-1E8 V-ATPase activity and pHi was significantly increased compared with those in pcDNA3 transfected and parental PC-3M-1E8 as the controls, and there was no difference among T1 and the controls. In order to confirm the specific influence of T2, T3, T4 variants to the V-ATPase activity and pHi, we first down-regulated the expression of ATP6V0C in PC-3M-1E8 cells by the transient transfection of the effective siRNA of ATP6V0C. Then the T1, T2, T3, and T4 variants were exogenously expressed in those cells. The pHi in T1, T2, T3, and T4 variants transfected PC-3M-1E8 cells and the controls showed no significant difference. The pHi was not increased in the T2, T3, and T4 variants transfected cells after the down-regulated expression of ATP6V0C (Fig. 5C and D).

ATP6V0C AND FLAG IN T2, T3, AND T4 TRANSFECTED CELLS COULD CO-IMMUNOPRECIPITATE

Since ATP6V0C was reported to bind directly to LASS2/TMSG1, immunoprecipitation was applied to identify the interaction between ATP6V0C and the different domains of LASS2/TMSG1. Western blot analysis of the immunoprecipitates identified the co-precipitation of exogenous ATP6V0C and FLAG in T2, T3, and T4 transfected HEK293T cells containing Homeodomain rather than in T1 transfected cells with Homeodomain deletion using anti-FLAG immunosorbent; ATP6V0C was not detected when mouse IgG replaced the anti-FLAG immunosorbent. In 12% SDS–PAGE, ATP6V0C migrated to the position of 16 kDa. The result was the first to provide convincing evidence that the Homeodomain of LASS2/TMSG1 was the direct interacting domain with ATP6V0C up till now (Fig. 6A–D).

FRET EFFICIENCY CONFIRMED THE INTERACTION OF ENDOGENOUS ATP6V0C AND EXOGENOUS FLAG COUPLED T2, T3, AND T4 RECOMBINED PROTEINS

Under Leica TCS SP5 fluorescence microscope, the majority of FLAG fusion proteins were localized to cytoplasm taking on green color with granular distribution and with a small proportion appearing in the nucleus, while ATP6V0C protein was mainly expressed in



Transfectant	FIR	PHi
1E8(T1)	7. 23±0. 76	6. 49
1E8(T2)	14. 22±1. 03	8. 79
1E8(T3)	13. 99±1. 66	8. 73
1E8(T4)	15. 12±0. 82	9. 03
1E8(pcDNA3)	6. 72±0. 59	6. 78
1E8	6. 25±0. 41	6. 66

Fig. 3. Evaluation of V-ATPase activity and intracellular PH. A: V-ATPase activity in T2, T3, and T4 variants transfected PC-3M-1E8 cells prominently increased in comparison with T1 variant transfected cells and the control ($^{*}P < 0.05$). B: The regression equation was $Y = 0.207X + 4.994$ obtained from calibration curve calculated by pHi (6.5, 7.0, 7.5, 8.0) and corresponding FIR of BCECF/AM fluorescence density detected by fluorospectrophotometer. C: The pHi of T2, T3, T4 variants transfected PC-3M-1E8 were significantly increased compared with those in T1 variant transfected cells and the controls evaluated from the regression equation $Y = 0.207X + 4.994$. D: Laser confocal microscope showed the fluorescence density of T2, T3, and T4 variants transfected PC-3M-1E8 cells were significantly decreased compared with those of T1 variant transfected and the controls ($^{*}P < 0.05$). [A, D: 1, PC-3M-1E8 (T1), 2, PC-3M-1E8 (T2), 3, PC-3M-1E8 (T3), 4, PC-3M-1E8 (T4), 5, PC-3M-1E8 (pcDNA 3), 6, PC-3M-1E8].

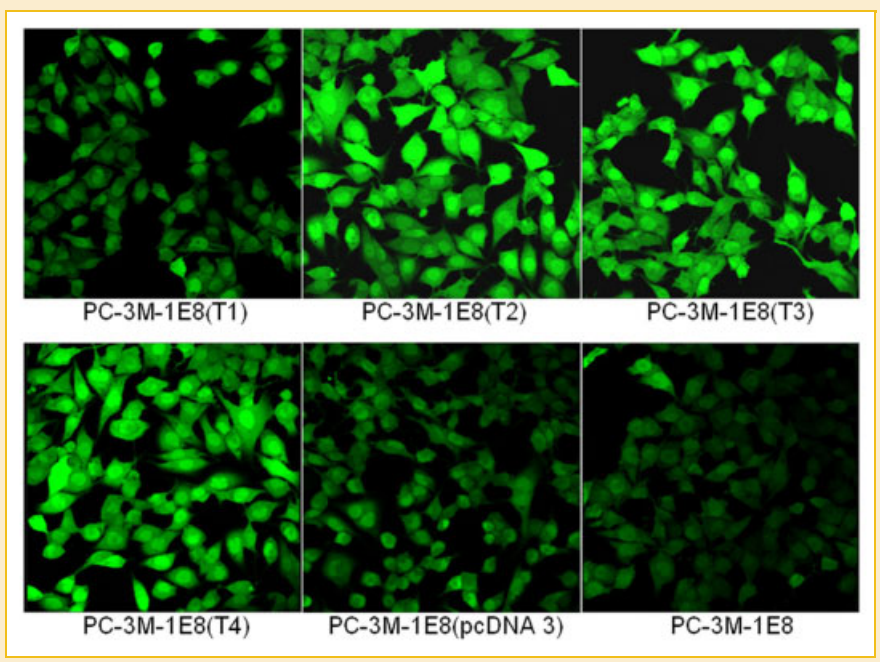


Fig. 4. The fluorescence intensity showed by BCECF/AM staining. Under laser confocal microscope, the fluorescence intensity in T2, T3, and T4 variants transfected PC-3M-1E8 cells were significantly weaker compared with those in T1 variant transfected and the controls.

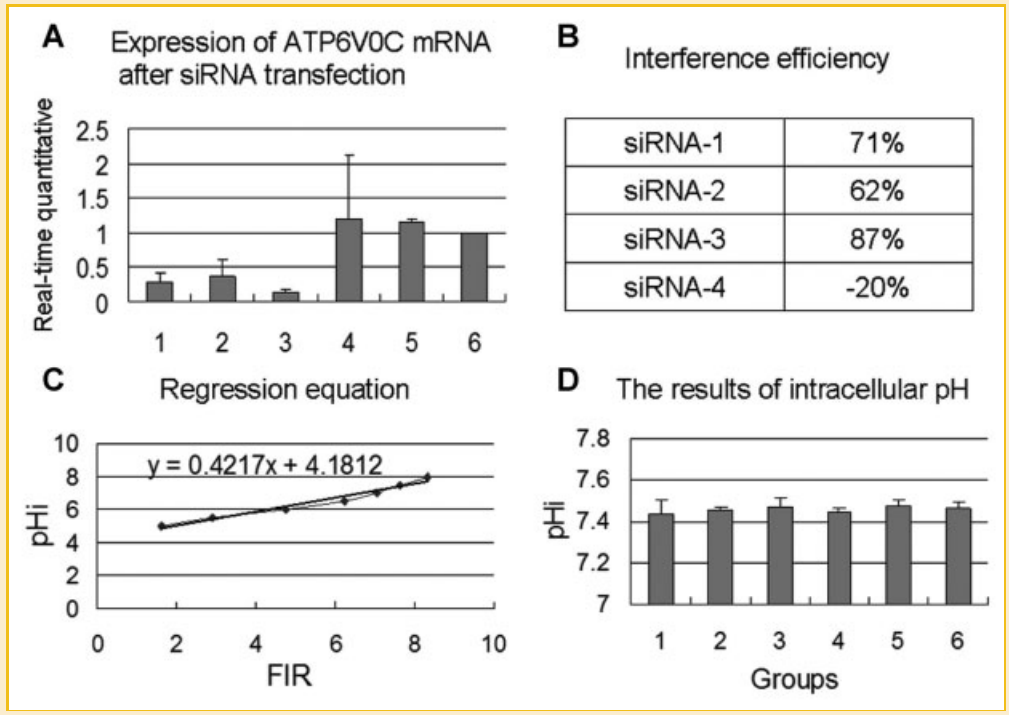


Fig. 5. A: The effects of ATP6V0C siRNAs on the mRNA expression of ATP6V0C in PC-3M-1E8 cells, and B: the interference efficiency of four siRNAs. C, D: The detection of intracellular pH after transfection of T1, T2, T3, and T4 variants in PC-3M-1E8 cells in which the C subunit of z-ATPase was down-regulated by siRNA: the regression equation was Y = 0.4217X + 4.1812, and the pHi of T2, T3, T4 variants transfected PC-3M-1E8 were not significantly increased compared with those in T1 variant transfected cells and the controls.[A: 1, siRNA-1, 2, siRNA-2, 3, siRNA-3, 4, siRNA-4, 5, Negative Control siRNA, 6, parental PC-3M-1E8. D: 1, PC-3M-1E8 (siRNA+T1), 2, PC-3M-1E8 (siRNA+T2), 3, PC-3M-1E8 (siRNA+T3), 4, PC-3M-1E8 (siRNA+T4), 5, PC-3M-1E8 (siRNA+pcDNA 3), 6, PC-3M-1E8 (siRNA)].

cytoplasm presenting on red color with extensive distribution. The merged views presented yellow color and they showed weaker yellow color in T1 variant transfected cell compared with others (Fig. 7A). The statistical results of FRET efficiency showed significant discrepancy between T1 transfected and other three variants transfected PC-3M-1E8 cells, which demonstrated that the T1 variants of LASS2/TMSG1 could not interact with ATP6V0C consistent to the results of immunoprecipitation assay (Fig. 7B).

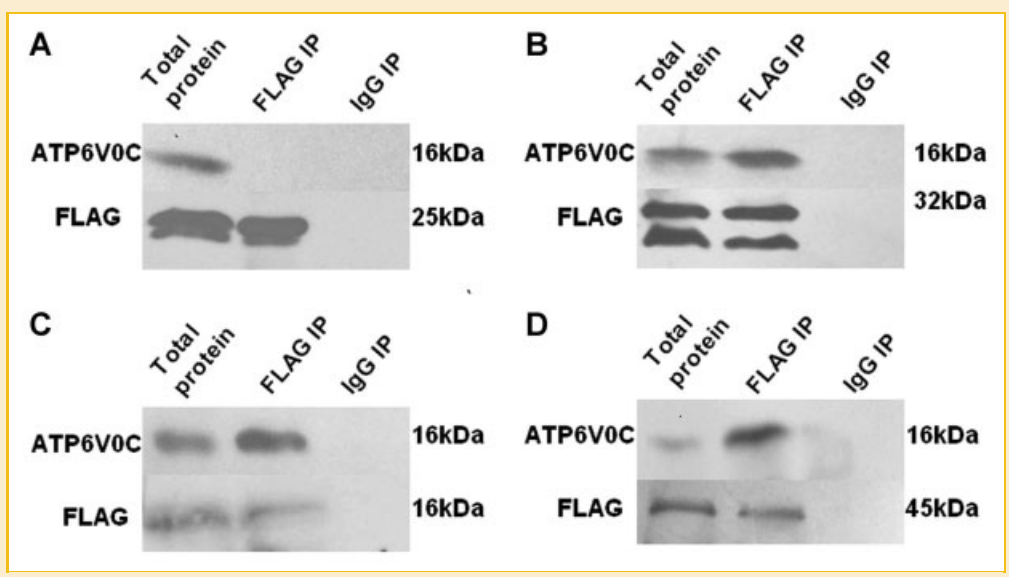


Fig. 6. Results of Immunoprecipitation using anti-FLAG immunosorbent. A: ATP6V0C could be detected in the exogenous ATP6V0C and T2, T3, or T4 variants cotransfected HEK293T cells rather than in T1 variant cotransfected cells with homeodomain deletion (A: ATP6V0C and T1 variant cotransfected HEK293T. B: ATP6V0C and T2 variant cotransfected HEK293T. C: ATP6V0C and T3 variant cotransfected HEK293T. D: ATP6V0C and T4 variant cotransfected HEK293T).

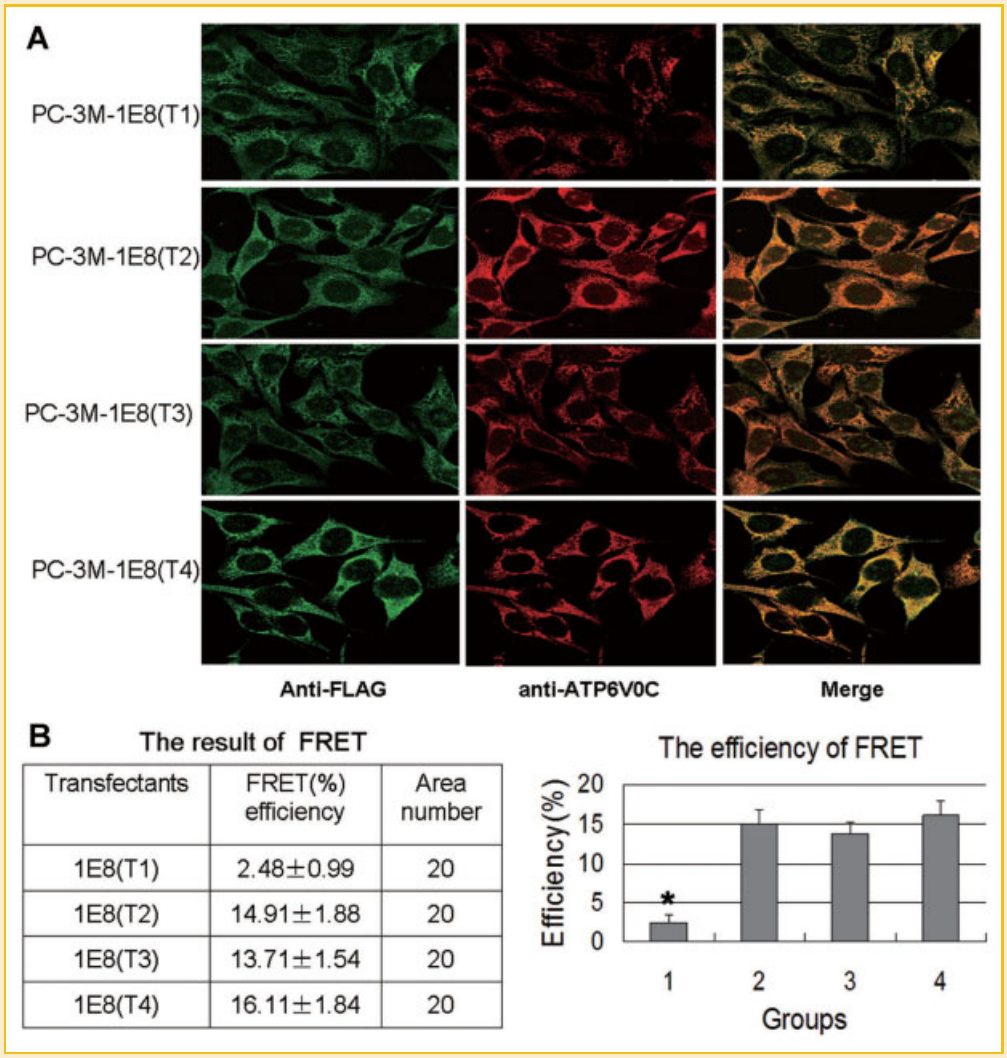


Fig. 7. The results of immunofluorescence and FRET. A: The majority of FLAG fusion proteins were localized to cytoplasm taking on green color with granular distribution and with a small proportion appearing in the nucleus, while ATP6V0C proteins were mainly expressed in cytoplasm presenting on red color with extensive distribution. The merged views showed weaker yellow color in T1 variant transfected cell compared to the other cells. B: The statistical results of FRET SE efficiency showed significant discrepancy between T1 transfected and other three variants transfected PC-3M-1E8 cells ($^{*}P < 0.05$). Representative of 20 areas in at least three independent experiments.

ELECTRON MICROSCOPE IMMUNOCYTOCHEMISTRY ANALYSIS CONFIRMED THE INTERACTION OF T2, T3, AND T4 VARIANTS OF LASS2/TMSG1 WITH ATP6V0C

Based on the results of immunoprecipitation and immunofluorescence, electron microscope immunocytochemistry analysis further intuitionally provided convincing evidence for the interaction of T2, T3, and T4 variants of LASS2/TMSG1 with ATP6V0C. In T1 transfected PC-3M-1E8 cells, the gold granules of 10 and 15 nm scattered separately mainly in the cytoplasm including endocytoplasmic reticulum and mitochondrial, and occasionally in the nucleus. In contrast, the two gold granules of different sizes tended to appear coupled in T2, T3 and T4 variants transfected PC-3M-1E8 cells. There was no gold granule detected in the negative control groups (Fig. 8).

ANALYSIS OF PROLIFERATIVE ABILITY OF TUMOR CELLS

To reveal the biological behaviors of prostate cancer cells after the interaction of Homeodomain of TMSG-1 and ATP6V0C, first we performed MTT assay to examine the proliferative ability of PC-3M-1E8 that exogenously expressed T1, T2, T3, and T4 variants. The results showed that PC-3M-1E8 expressed exogenous T1 grew more slowly than pcDNA3-transfected cells and parental cells, but more quickly than cells expressed exogenous T2, T3, and T4 variants under the same circumstance ($P < 0.05$, Fig. 9A).

APOPTOSIS AND CELL CYCLE PROGRESSION ANALYSIS BY FLOW CYTOMETRY

The previous study of our group revealed that the over expression of TMSG-1 in PC-3M-1E8 could prompt apoptosis of tumor cells [Su et al., 2008]. Therefore, we detected the apoptosis of PC-3M-1E8 that exogenously expressed T1, T2, T3, and T4 variants. The apoptosis rate of T1 transfected cells was higher than those of the controls ($P < 0.05$), but lower than cells over-expressed T2, T3, and T4 variants ($P < 0.05$). In cells over-expressed T2, T3, and T4 variants,

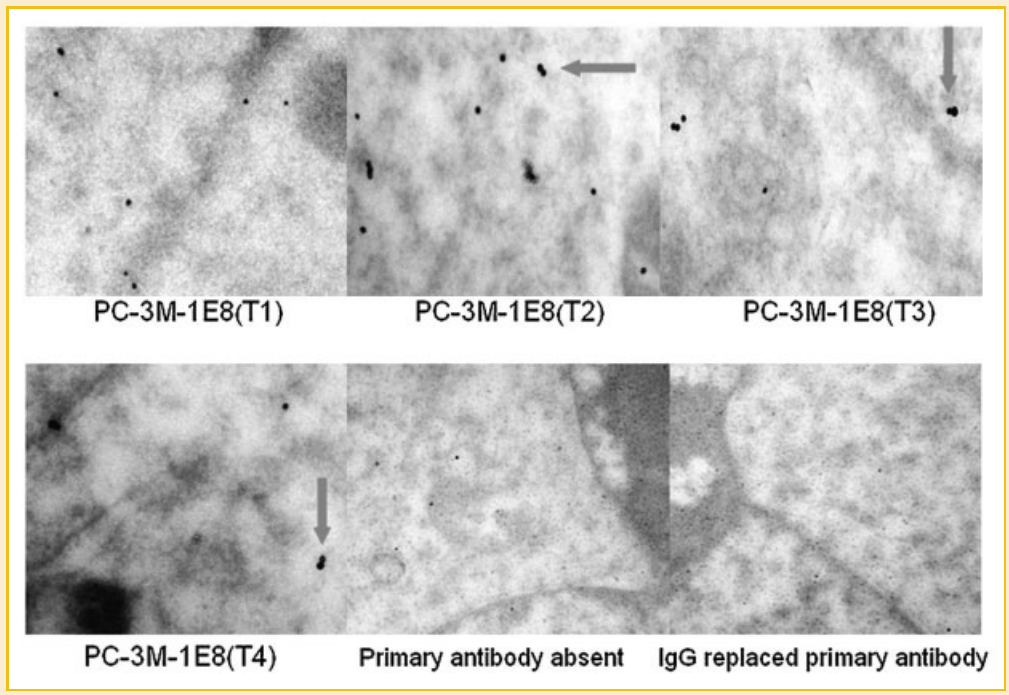


Fig. 8. The two gold granules of different sizes tended to appear coupled in T2, T3, and T4 variants transfected PC-3M-1E8 cells rather than in T1 variant transfected cells (gray arrow). There was no gold granule detected in the negative control groups (primary antibody absent or IgG replaced primary antibody).

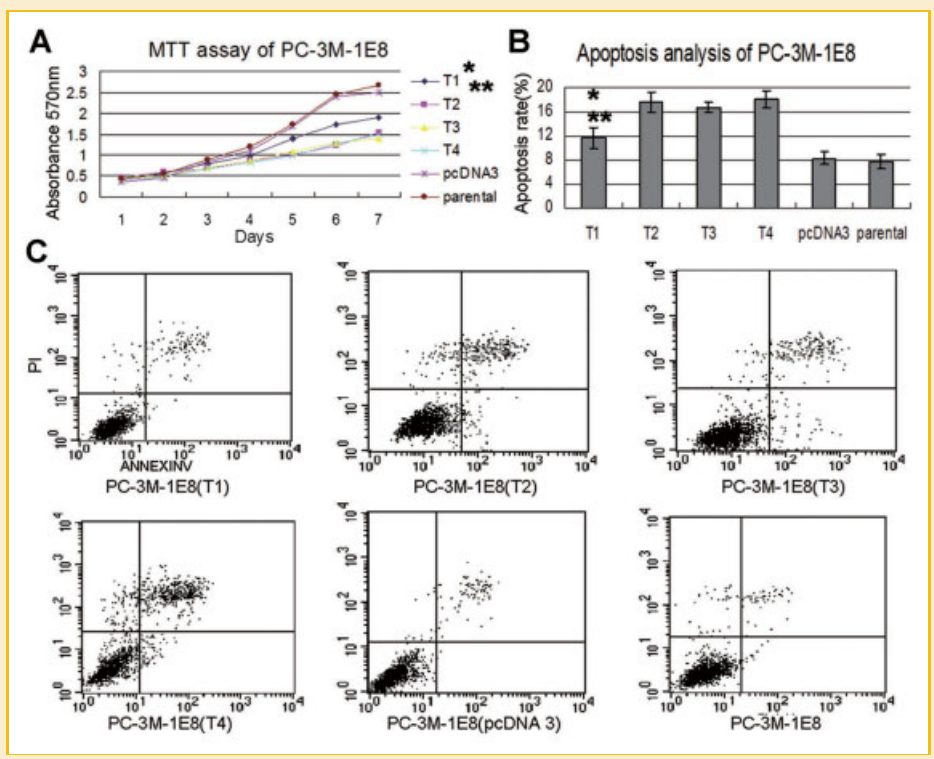


Fig. 9. Analysis of proliferation ability and apoptosis of tumor cells. A: MTT assay showed PC-3M-1E8 expressed exogenous T1 grew more slowly than the controls ($^{*}P < 0.05$), but more quickly than cells expressed exogenous T2, T3, and T4 variants under the same circumstance ($^{**}P < 0.05$). B, C: The apoptosis rate of T1 transfected cells was higher than those of the controls ($^{*}P < 0.05$), but lower than cells over-expressed T2, T3, and T4 variants ($^{**}P < 0.05$). In cells over-expressed T2, T3, and T4 variants, the apoptosis rates did not show any significant difference, while all were higher than those in the controls.

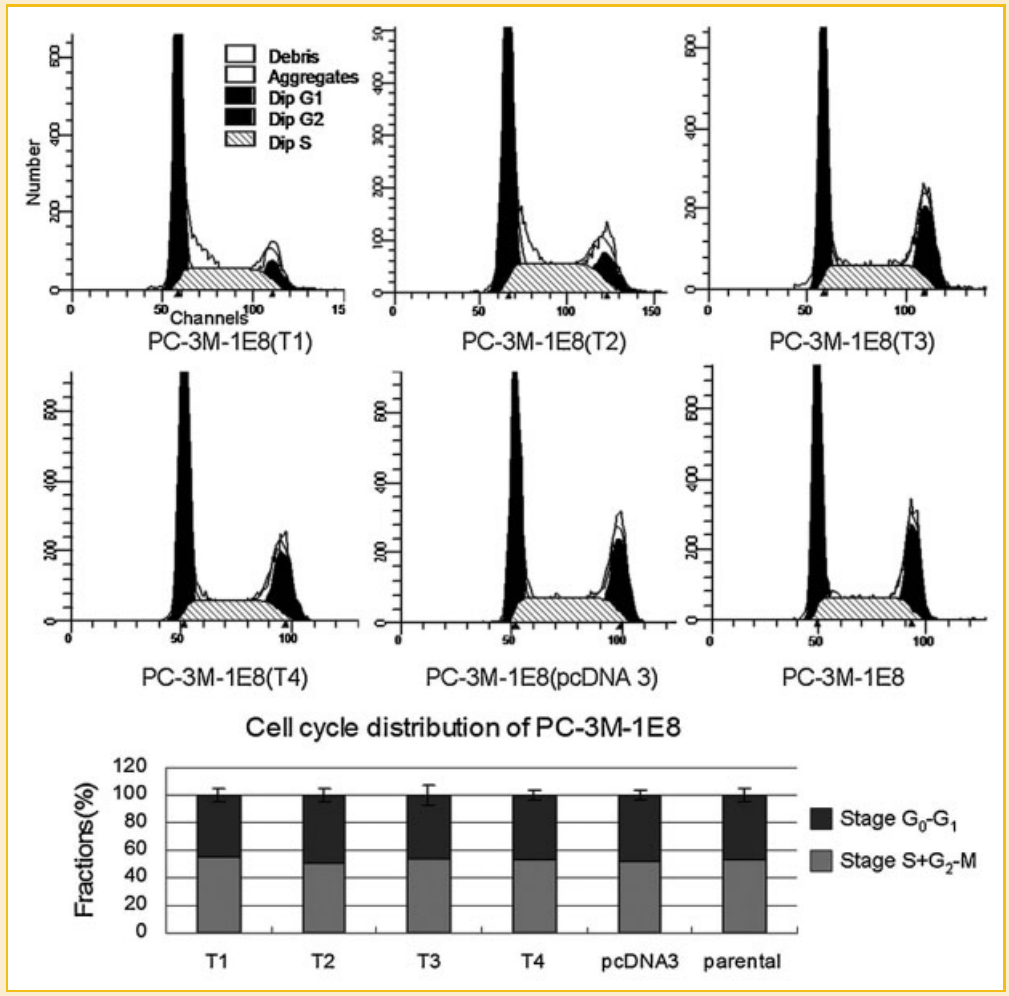


Fig. 10. Cell cycle progression of different groups. No significant difference was revealed between cells overexpressed T1, T2, T3, and T4 variants, the controls as well ($P > 0.05$).

the apoptosis rates did not show any significant difference, while all were higher than those in the controls (Fig. 9B and C).

Next we detected the cell cycle progression of different groups. However, no significant difference was revealed between cells over-expressed T1, T2, T3, and T4 variants, the controls as well ($P > 0.05$, Fig. 10).

EVALUATION OF TUMOR INVASIVE ABILITY

Matrigel invasion assay was performed to detect the influence on cell invasive ability after the interaction of Homeodomain of TMSG-1 and ATP6V0C. The cell numbers passed through the matrigel and membrane in the groups over-expressed T1, T2, T3, and T4 variants were less than those in the controls ($P < 0.05$). The cell numbers in T1 and T3 transfected cells were a bit more than those in T2 and T4 variants transfected cells, but no significant difference was revealed ($P > 0.05$, Fig. 11).

DISCUSSION

LASS2/TMSG1 gene was first cloned as a novel tumor metastasis suppressor gene by our laboratory from non-metastatic and metastatic cancer cell variants of human prostate carcinoma by mRNA differential display in 1999 [Ma et al., 2002]. Our former 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 [Fei et al., 2004]. Using sense and antisense cDNA transfection, LASS2/TMSG1 was found to suppress the invasion ability significantly 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 [Su et al., 2007, 2008]. It was notable that LASS2/TMSG1 associated with subunit C of V-ATPases [Pan et al., 2001].

However, as a novel tumor metastasis suppressor gene, the precise molecular mechanisms of LASS2/TMSG1 on cancer metastasis through V-ATPase are unclear. Which domain is the direct interaction between LASS2/TMSG1 and subunit C of V-ATPases?

In an attempt to understand the mechanism through which LASS2/TMSG1 interacting with ATP6V0C in addition to regulating the intracellular pHi, we first cloned four variants containing different domains of LASS2/TMSG1 into pcDNA3 and stably

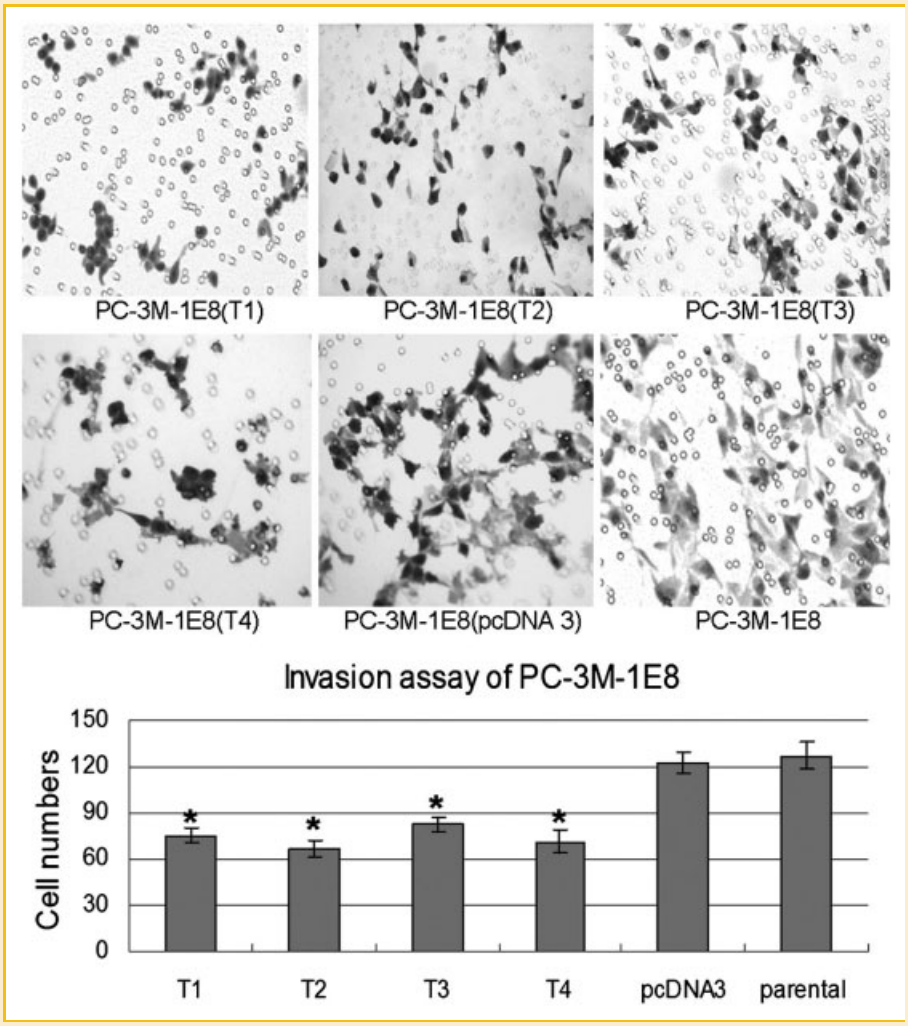


Fig. 11. Evaluation of tumor invasive ability. The cell numbers passed through the matrigel and membrane in the groups over-expressed T1, T2, T3, and T4 variants were less than those in the controls ($^{*}P < 0.05$). The cell numbers in T1 and T3 transfected cells were a bit more than those in T2 and T4 variants transfected cells, but no significant difference was revealed ($P > 0.05$).

transfected the variants to PC-3M-1E8 cell. QPCR and Western blot analysis did not present any variation of ATP6V0C expression in mRNA and protein levels after transfection. Despite no change of the amount of ATP6V0C, V-ATPase activity and intracellular pHi increased obviously after the transfection of variants containing the Homeodomain of LASS2/TMSG1 (T2, T3, and T4). It was indicated that up regulation of the Homeodomain of LASS2/TMSG1 in PC-3M-1E8 cells could promote the activity of V-ATPase. Then we detected that after the C subunit of V-ATPase was down-regulated in cells by siRNA, the intracellular pH was not increased after transfection of T1, T2, T3, and T4 variants. It further confirmed that the Homeodomain of LASS2/TMSG1 was very likely associated with the promotion of V-ATPase activity and the increase of intracellular pH.

Based on the aforementioned results, immunoprecipitation was carried out. In the initial study, we transfected the variants to PC-3M-1E8 cells. However, the co-immunoprecipitation failed attributed to low expression level of FLAG in PC-3M-1E8 cells in addition to the limited sensitivity of the ATP6V0C antibody. After the co-transfection of the variants and ATP6V0C to HEK293T cells, it was intriguing in the T2, T3, and T4 variants containing Homeodomain transfected HEK293T cells, the co-immunoprecipitation of FLAG and ATP6V0C were clearly detected. Nevertheless, the T1 fragment, which only contains the TLC domain of LASS2/TMSG1 with the deletion of Homeodomain could not co-immunoprecipitate with ATP6V0C.

Taking into account the discretion of the conclusion, we further applied indirect immunofluorescence by double staining and FRET analysis and electron microscope immunocytochemistry analysis in order to observe the interaction of FLAG and ATP6V0C intuitionally. The results of immunofluorescence and electron microscope strikingly confirmed the results of immunoprecipitation that the T1 fragment with the deletion of Homeodomain could not coexist with ATP6V0C. Furthermore, the most surprising characteristic of LASS2/TMSG1 is that biochemical studies predict them to encode transmembrane proteins, with the Homeodomain on the cytosolic

side of the endoplasmic reticulum membrane, and hence they could not act as DNA-binding proteins or transcription factors [Mizutani et al., 2005; Pewzner-Jung et al., 2006]. Besides, V-ATPase is ubiquitous proton-translocating pumps located in membranes of vacuoles, lysosomes and other components of the endomembrane system [Pan et al., 2001]. Thus the membrane localization is ideally positioned to facilitate the interaction between LASS2/TMSG1 and subunit C of V-ATPase (V-ATPase). Based on the results of our study and the literature, we concluded that the over expression of Homeodomain could combine ATP6V0C to the membrane to facilitate the activation of V-ATPase. The Homeodomain of LASS2/TMSG1 could not only be taken as a transcriptional factor but also play important roles in tumor proliferation and metastasis through interacting with ATP6V0C.

Moreover, our results showed that loss of Homeodomain markedly enhanced the proliferation ability but weakened the apoptotic effect of LASS2/TMSG1 in PC-3M-1E8 cells. The explanation is as follows. Spontaneous apoptosis usually happened under the condition of intracellular alkalinization and seldom in acidification [Zhu and Loh, 1995]. The Homeodomain of LASS2/TMSG1 could increase the activity of V-ATPase by directly binding ATP6V0C, accelerated the proton transmembrane secretion and thus decreased intracellular H^+ concentration, and then lead to the increase of intracellular pH, which was reported to be related to increasing apoptosis of cells and thus decreasing the cell numbers [Lu et al., 2005].

In summary, LASS2/TMSG1 could increase the activity of V-ATPase by directly binding ATP6V0C through its Homeodomain, then increase of intracellular pH, and eventually lead to apoptosis of tumor cells, which perhaps was one potential mechanism of tumor metastasis suppression. However, we did not found significant difference about the invasive ability between different variants over-expressed cells. This might because that the Homeodomain and TLC domain both could contribute to influence the invasive ability of tumor cells. The complicated mechanisms need further studies.

In conclusion, our study for the first time confirmed 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 to influence the apoptosis and proliferation of tumor cells, which provided important clues for understanding the mechanism of LASS2/TMSG-1 on the behavior of tumor cells.

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