The Growth and Metastasis of Human Hepatocellular Carcinoma Xenografts Are Inhibited by Small Interfering RNA Targeting to the Subunit ATP6L of Proton Pump

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Abstract

Extracellular pH is usually low in solid tumors, in contrast to the approximately neutral intracellular pH. V-ATPase, which plays an important role in maintaining neutral cytosolic pH, very acidic luminal pH, and acidic extracellular pH, is a proton pump. ATP6L, the 16 kDa subunit of proton pump V-ATPase, can provide proton hydrophilic transmembrane path. In this study, ATP6L in a human hepatocellular carcinoma cell line with highly metastatic potential (HCCLM3) was knocked down using DNA vector–based small interfering RNA (siRNA) to suppress the metastasis. The expression of ATP6L in stable siRNA transfectants, designated as si-HCCLM3 cells, was inhibited by ~60%. The proton secretion and the intracellular pH recovery from NH4Cl-prepulsed acidification were inhibited in si-HCCLM3 cells. The invasion of the si-HCCLM3 cells was suppressed in vitro; simultaneously, the expressions of matrix metalloproteinase-2 and gelatinase activity were reduced. In vivo, at 35th day after implantation of the si-HCCLM3 xenografts into the livers in BalB/c (nu+/nu+) mice, the size of liver tumor tissues was dramatically smaller in siRNA group than in the controlled group. The most impressive effect of ATP6L siRNA is its striking reduction of the metastatic potential of HCCLM3 cells. In control, all eight mice had the intrahepatic metastasis and six of eight the pulmonary metastasis, whereas in ATP6L siRNA-treated group, three of eight had the intrahepatic metastasis and only one of eight the pulmonary metastasis. The results suggest that the inhibition of V-ATPase function via knockdown of ATP6L expression using RNA interfering technology can effectively retard the cancer growth and suppress the cancer metastasis by the decrease of proton extrusion and the down-regulation of gelatinase activity. (Cancer Res 2005; 65(15): 6843-9)

Introduction

The increased glycolysis and proton secretion in tumors is proposed to contribute to the proliferation and invasion of cancer cells during the process of tumorigenesis and metastasis (1). The altered phenotype of glucose metabolism in cancer cells results in the excessive production of intracellular acidity. The intracellular pH (pHi) is a vital parameter for almost all biological processes in cells. It has been described that intracellular acidification is an inducing factor of apoptosis (2–4), and intracellular alkalization seems to be necessary for proliferation (4–6). Proton pump V-ATPase can pump protons into extracellular environment or lumens of some membrane-bound organelles involved in maintaining a relative neutral pHi and acidic luminal pH or extracellular pH (pHe). Recently, much attention has been paid to the role that the highly active V-ATPase plays in tumorigenesis and metastasis of cancer (9).

V-ATPase is expressed in plasma membrane of some tumor cells, which is correlated with the metastatic potential of these cells (10–12). The extrusion of proton causes extracellular acidification, which can up-regulate the proteases that digest extracellular matrix (ECM) during invasion and metastasis. Many members of the cathepsin family and matrix metalloproteinase (MMP) family are pH sensitive; in most cases, the low pH of extracellular microenvironment can induce the increase of the secretion of proteases and enhance the activation of the relevant proteases either in intracellular vesicles or in extracellular environment (13–17). Therefore, we could postulate that inhibition of the function of V-ATPase would affect the pHi and pHe, and consequently influence metastatic potential of cancer cells as well as their proliferation.

ATP6L, a subunit of V-ATPase complex, provides proton a hydrophilic path across the membrane. It has been reported that ATP6l protein is mildly to markedly expressed in invasive pancreatic cancers (42 of 46, 92%), but not detected in the noninvasive pancreatic cancers (11). In vitro, the transfected fibroblast cell line with overexpressing ATP6L behaves with increased invasion accompanied by up-regulation of secretion of MMP-2 (18). MMP-2 is closely related to cancer metastasis (19) and its activity is increased at the invasive borders of the metastatic tumor tissue of hepatocellular carcinoma (20), colon carcinoma (21, 22), or pancreatic carcinoma (22). In this report, we described that a DNA vector–mediated small interfering RNA (siRNA) technology (23, 24) was utilized to knock down ATP6L expression in highly metastatic HCCLM3 cells (25, 26) so as to suppress their growth and metastatic potential. Meanwhile, we elucidated the possible mechanisms relevant to the inhibition of cancer metastasis by investigating the activity of V-ATPase and the expression and activity of MMP-2 in the stable transfected cells.

Materials and Methods

Preparation of DMEM medium buffered by NaHCO3/CO2 system. The appropriate amount (2.1 g for 25 mmol/L NaHCO3 or 0.084 g for 1 mmol/L NaHCO3) of sodium bicarbonate was completely dissolved in the...
DMEM medium per liter (Life Technologies, Grand Island, NY). After the medium containing NaHCO₃ had been balanced for 5 hours in 5% CO₂ at 37°C, the pH of the DMEM medium buffered by NaHCO₃/CO₂ system was adjusted to the desired pH value monitored by pH240 pH/Temp Meter (Beckman Coulter, Inc., Fullerton, CA) under the same condition.

**Cells and cell culture.** Cell line HCCLM3 was established by Liver Cancer Institute, Zhongshan Hospital, Fudan University (25). Cells were cultured in HCO₃⁻-buffered DMEM medium [25 mmol/L NaHCO₃ (pH 7.0)], 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. Experiments were done in this culture condition, unless indicated otherwise.

**Construction of small interfering RNA plasmid vector.** The siRNA expressing vector pSilencer 2.1-U6 was purchased from Ambion, Inc. (Austin, TX). The two complementary oligonucleotides (5’-GATCCCAAGTC-CATCATCCACAGTTGCCTCAGGACTAGACCTGCGAT-GATGGACTTTTGTGAAAAG-3’ and 5’- AGCGTTTCTAAAAGCTGTTGCACTCGTC-3’; the 21-nucleotide sense or antisense strand is in bold letters, and stem loop sequences are in italics) were synthesized (BioAsia, Co., Shanghai), then annealed to generate double-stranded DNAs and ligated into the linearized empty vector pSilencer 2.1-U6. The siRNA plasmid encoded the hairpin RNAs that specifically targeted 390- to 410-nucleotide ATP6 mRNA (Genbank accession no. M62762) and had no significant homology with other known genes. As a negative control, the scrambled plasmid was designed to encode a hairpin RNA containing the same percentage of bp as siRNA plasmid, but in random sense sequence (AAGTCTGAGGTCATTGGAATGTGGGGACAGT-3’), and did not target any known gene. Plasmids were verified by sequence analysis (United Gene Holdings, Ltd., Shanghai, China).

**Transfection.** Transfection of plasmid DNAs into HCCLM3 cells was done using LipofectAMINE (Invitrogen, Carlsbad, CA). Briefly, 1 x 10⁵ cells were seeded per well into six-well plates (Corning, Inc., Corning, NY). The cells were transfected using 2 μg DNA mixed with 5 μL LipofectAMINE in 1 mL medium without serum and antibiotics. The cells were incubated for 24 hours, and then 1 mL medium containing 20% serum was added into each well. The cells were cultured and screened in the medium containing 10% serum and 3 mg/mL G418 (Invitrogen) for at least 2 weeks until the nontransfected HCCLM3 cells cultured in the controlled wells were all killed. The stable transfectants, obtained by transfecting the ATP6L siRNA plasmid or scrambled plasmid into the HCCLM3 cells, were designated as si-HCCLM3 cells or sc-HCCLM3 cells, respectively.

**Expression of metalloproteinase and activity of matrix metalloproteinase.** Total RNA from cultured cells was isolated using the TRIzol reagent (Invitrogen) and first-strand cDNA was synthesized using 2 μg total RNA and SuperScript II reagent (Invitrogen). The ATP6L or β-actin was amplified by PCR at conditions of 95°C for 1 minute; followed 26 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds; then 72°C for 10 minutes in the presence of Taq polymerase (TaKaRa Bio, Inc., Tokyo), 10 pmol/μL of forward 5’-GGATCTGCTGCTGTCGAT-TG-3’ and reverse 5’-GGCTATGTGTCAGGTGTTCG-3’ primers for ATP6L, or 10 pmol/μL of forward 5’-AAGTACCTGGTGATGATGC-3’ and reverse 5’-TCAAGTGTTGGGGACAAAG-3’ primers for β-actin. A 948 bp ATP6L cDNA, as probe for Northern blotting, was obtained by yeast two-hybrid screening as ATP6L mRNA signal band intensities was done using the imaging analysis software (SmartView version 5.0, FURJ Science and Technology, Co., Ltd., Shanghai, China).

**In vitro cell invasion.** Cell invasion assay was done using Cell Invasion Assay kit (Chemicon, Temecula, CA) according to the manufacturer's instructions with some modifications. The invasion of HCCLM3 cells in medium with a series pH values was measured. Briefly, HCCLM3 cells were cultured in HCO₃⁻-buffered DMEM medium (pH 7.0) containing 10% FBS, reached 80% confluence, and were then starved in serum-free medium for 16 hours. Cells were washed with 0.9% NaCl twice, harvested, and diluted to the concentration of 1 x 10⁶/mL in serum-free DMEM medium with a series of pH ranging from 6.0 to 8.0 buffered by HCO₃⁻ (1 mmol/L NaHCO₃). Then, cells in different pH medium were added into invasion chamber (1 x 10⁵ cells in 100 μL medium per well). The invasion chamber was inserted into the feeder tray, which contained 150 μL of 10% FBS HCO₃⁻-buffered DMEM medium per well, with pH corresponding to the inserting well of the upper invasion chamber. The plate was incubated for 20 hours at 37°C in 5% CO₂. The cells that had invaded through ECMatix-coated membrane was washed with detachment solution, stained with lyso buffer/dye solution, and the fluorescence absorbance was measured in a Perkin-Elmer Luminescence Spectrometer (LS 50B) at 480/520 nm filter set.

The invasion of HCCLM3 cells, sc-HCCLM3 cells, or si-HCCLM3 cells cultured in HCO₃⁻-buffered DMEM medium [1 mmol/L NaHCO₃ (pH 7.0)] was measured as described above.

**Activity of V-ATPase.** pH and pHi were measured using pH-sensitive dye BCECF (Sigma Chemical Co., St. Louis, MO). The ability of proton secretion was determined by measuring pH/e. Cells (3 x 10⁵) were seeded per well in 96-well plate and were cultured in HCO₃⁻-buffered DMEM medium (pH 7.0) containing 5% FBS at 37°C in 5% CO₂ for about 5 hours. The medium was removed after the cells attached to the plate. The cells were washed twice using 0.9% NaCl, 120 μL serum-free HCO₃⁻-buffered DMEM medium [1 mmol/L NaHCO₃ (pH 7.0)] was added into each well, and cells were incubated at 37°C in 5% CO₂. After the cells were cultured for 4, 8, or 12 hours, respectively, 100 μL supernatant per well was collected and 1 μL of BCECF was added into each sample. The sample was excited at 490 and 440 nm and the emitted fluorescence was measured at 535 nm by the Perkin-Elmer LS-50B. pH was calibrated with the curve plotted by the fluorescence ratio F900/F440 of standard DMEM medium containing 1 μmol/L BCECF with a series of pH buffered by HCO₃⁻, pH/e value was converted to the extracellular proton concentration.

pH recovery from NH₄Cl-prepulsed acidification was observed and real-time pH/e was monitored using BCECF-“Fast Filter” accessory in the PE luminescence spectrometer, as previously described (28–30). Briefly, cells were grown on coverslips (Fisher Scientific, Fair Lawn, NJ) to ~80% confluence in the HCO₃⁻-buffered DMEM medium (pH 7.0) containing 10% FBS at 37°C in 5% CO₂ and were incubated in the same medium containing 1 μmol/L BCECF-AM (Sigma) for 20 minutes. The above BCECF-loaded monolayer cells were balanced in solution A [140 mmol/L N-methyl-D-glucamine chloride (Sigma), 5 mmol/L KCl, 1 mmol/L MgCl₂, 10 mmol/L glucose, 10 mmol/L HEPES (pH 7.2)]. The cells were then exposed to solution B [50 mmol/L KCl, 10 mmol/L MgCl₂, 10 mmol/L glucose, 10 mg/mL balfilomycin A1 (LC Laboratories, Woburn, MA)]. pH/e values were measured with high-K⁺ nigericin technique as previously reported (31, 32).

**Expression of matrix metalloproteinase-2 and activity of matrix metalloproteinase-2/matrix metalloproteinase-9.** MMP-2 expression in cells was assayed by Western blot. Cells (5 x 10⁵) were planted in 6 cm dishes and cultured in HCO₃⁻-buffered DMEM medium (pH 7.0) containing 5% FBS for 24 hours. Then, the cells were harvested using Tissue Protein Extraction Reagent (Pierce Chemical, Co., Rockford, IL). The protein concentration was determined using the BCA method (Pierce). Ten micrograms of protein from each sample was added to each lane, separated in 10% SDS-PAGE, and then transferred to membranes. The membranes were incubated overnight at 4°C in PBST and 5% dry milk containing monoclonal mouse anti-human–MMP-2 antibody (1:400, Chemicon), or monoclonal mouse anti-human–β-actin (Sigma) as a control. The membranes were then washed and incubated for 2 hours at room temperature in PBST and 5% dry milk containing a horseradish peroxidase–conjugated goat anti-mouse secondary antibody (1:1,000, Sigma), washed and incubated for 5 minutes with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce), and then exposed to X-ray films.

Activity of gelatinase in the supernatant of the cultured cells was measured with MMP Gelatinase Activity Assay kit (Chemicon). Cells (1.5 x 10⁵ per well) were plated in triplicate in 24-well plate and cultured for 24 hours in HCO₃⁻-buffered DMEM medium (pH 7.0) containing 5% FBS at
37°C in 5% CO₂. Then, cells were washed with 0.9% NaCl twice and 0.5 mL HCO₃⁻-buffered serum-free DMEM medium [1 mmol/L NaHCO₃ (pH 7.0)] was added into each well and incubated at 37°C in 5% CO₂ for 24 hours. The supernatant was collected, the protein concentration was determined using the BCA method (Pierce), and the supernatant was diluted to 1 mg/mL. Gelatinase activity was quantified according to the manufacturer's instruction with some modifications. Ten-microliter aliquots of each sample were incubated with diluted biotinylated-gelatinase substrate for 4 hours. The mixture was then transferred into biotin-binding plate and continuously incubated for 30 minutes at 37°C. After the plate had been washed, streptavidin-enzyme conjugate was added to each well, incubated for 30 minutes at 37°C, and then washed. Substrate solution was added and incubated at room temperature for 10 minutes and absorbance at 450 nm was determined. The activity of sample = (absorbance of standard MMP-2/MMP-9) / (absorbance of sample).

Zymography of supernatant was done as previously reported (21). Each sample of supernatant containing 20 μg protein was fractioned on 10% SDS-PAGE containing 25 μg/mL FITC-labeled DQ-gelatin (EnzChek, Molecular Probes, Eugene, OR). After electrophoresis, gels were washed for 30 minutes in washing buffer [50 mmol/L Tris-HCl, 2.5% Triton X-100, 5 mmol/L CaCl₂ (pH 7.5)] and incubated in buffer [50 mmol/L Tris-HCl, 1% Triton X-100, 5 mmol/L CaCl₂ (pH 7.5)] for 20 hours. The gel was observed on Amersham Typhoon 9400 imager.

In situ gelatinase zymography of xenografts was observed as described previously (21). One percent of low gelling temperature agarose (Invitrogen) was added into each well and incubated at 37°C for 40°C, and poured on the cryostat sections of the tumor tissues removed from the livers of the xenografted mice. After the agarose was gelled at 4°C, the sections were incubated at room temperature for 1 hour. Fluorescence was observed with excitation at 460 to 500 nm and emission at 512 to 542 nm on a Bio-Rad Laser Scanning Confocal Microscope (Radiance 2100).

Xenograft model. Metastasis model on mice was made as described previously (25). Cells (5 × 10⁶) of HCCLM3, scr-HCCLM3, or si-HCCLM3 were s.c. injected into the left upper flank regions of three experimental mice (4-6 weeks of age, male, BalB/c nu +/nu+, from Shanghai Cancer Institute) respectively. At the 28th day, when the size of s.c. tumor had reached ~1 cm in diameter, the mice were killed and the tumor tissues were removed into aseptic PBS. Then, s.c. tumor tissues were cut into slices (~1 × 1 × 1 mm³) and implanted into livers of three groups of nude mice, with eight mice in each group. The animals were killed after 35 days. Size of local tumors at the implanting site in liver was calculated by measuring the length, width, and thickness with a caliper. Volume of tumor was calculated as follows: tumor volume (mm³) = length × width × thickness. Relative tumor volume = (volume of tumor tissue) / (volume of initially planted tumor tissue). Mice whose small tumor colonies were seen with naked eyes around the tumor nodule derived from the implanted tumor tissue at the implantation site were considered as in situ metastasis positive. Then, the local tumors were frozen and sectioned for assay of MMP-2/MMP-9 zymography in situ (see above). The lung tissue of each mouse was fixed, embedded, sectioned serially, stained with H&E, and observed under a microscope. Mice whose metastatic hepatocellular carcinoma cells were found on any slide of lung sections were considered as lung metastasis positive. The expression of beta-actin was examined in all samples using the method described by Maw et al. (8). The experiment was repeated in 10 randomly selected tumors. The expression of beta-actin was examined in all samples using the method described by Maw et al. (8). The experiment was repeated in 10 randomly selected tumors.

Results

Suppression of ATP6L expression by siRNA. HCCLM3 cells, the hepatocellular carcinoma cells with highly metastatic potential, were transfected with the constructed plasmid encoding siRNA targeting to ATP6L and with negative controlled plasmid containing scrambled random sequence, respectively. The stable transfectants with ATP6L siRNA (si-HCCLM3) and scrambled random sequence siRNA (scr-HCCLM3) were established by G418 screening. To examine the efficiency for silencing ATP6L, we detected the level of mRNA expression in the transfectants. The reverse transcription-PCR (RT-PCR) data showed that the level of ATP6L mRNA was apparently decreased in si-HCCLM3 cells compared with HCCLM3 cells, but there was no difference between the scr-HCCLM3 cells and HCCLM3 cells (Fig. 1A). Furthermore, the result of RT-PCR was confirmed by Northern analysis using the ³²P-labeled ATP6L cDNA as a probe. The quantitative analysis of ATP6L band on Northern blotting showed that the level of mRNA of ATP6L in si-HCCLM3 was decreased by ~60%, whereas the negative controlled plasmid did not affect the expression of ATP6L mRNA (Fig. 1B).

Inhibition of in vitro invasion. We used the Invasion Assay kit to explore the correlation between in vitro invasion of the HCCLM3 cells and pH of the medium. The results showed that the invasion ratio of HCCLM3 cells was higher in acidic pH (Fig. 2A). Meanwhile, the invasion of HCCLM3 cells, scr-HCCLM3 cells, or si-HCCLM3 cells was compared. The invasion ratio of si-HCCLM3 cells was remarkably decreased compared with HCCLM3 cells, whereas that of scr-HCCLM3 cells was not inhibited (Fig. 2B). The data indicated that knockdown of ATP6L expression could inhibit the invasion of HCCLM3 in vitro. Considering that HCCLM3 cells were more invasive in the environment of acidic pH, the suppression of invasion of si-HCCLM3 cells should be related to the inhibition of proton secretion in the cells.

Inhibition of V-ATPase activity. The process of proton extrusion was shown by detection of proton concentration in medium with pH-sensitive BCECF. As shown in Fig. 3A, the proton secretion of si-HCCLM3 cells was notably reduced at 12 hours, whereas that of scr-HCCLM3 cells was not affected compared with that of HCCLM3 cells.

pH recovery from intracellular acidification induced by NH₄Cl prepulse is shown in Fig. 3B-D. When exposed to NH₄Cl, pH recovered rapidly and then decreased gradually. After the removal of NH₄Cl, pH dropped rapidly. In the following procedure, the pH in HCCLM3 cells or scr-HCCLM3 cells recovered, whereas it hardly recovered in si-HCCLM3 cells. Under the Na⁺- and HCO₃⁻-free condition, pH in HCCLM3 cells or scr-HCCLM3 cells recovered, and the recovery could be blocked in the presence of bafilomycin.

Inhibition of Cancer Metastasis by ATP6L siRNA

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A1. The above results revealed that the pHi recovery was due to active V-ATPase.

The suppression of V-ATPase concomitant with inhibition of gelatinase. The secretion and activation of some ECM-degrading proteases are pH regulated. We determined the expression level and activity of MMP-2, which is closely related to cancer metastasis according to the previous reports (19, 20). Western blot showed that the expression of MMP-2 was down-regulated in si-HCCCLM3 cells compared with HCCCLM3 cells (Fig. 4A).

Furthermore, the supernatant of cultured cells was collected and the gelatinase activity was assayed with MMP-2/MMP-9 Activity Assay kit. The activity of gelatinase was apparently reduced in the supernatant of si-HCCCLM3 cells compared with HCCCLM3 cells (Fig. 4B). In addition, zymography showed that MMP-2/MMP-9 activity was suppressed in si-HCCCLM3 cells (Fig. 4C).

Moreover, the activated gelatinase in xenografts was examined by *in situ* zymography. In the frozen sections of tumor tissues, the gelatinolytic activity was observed around the cells and in the cytoplasm of the cells. Gelatinase activity of xenografts in si-HCCCLM3 group was inhibited (Fig. 4D).

Growth retardation of si-HCCCLM3 xenografts. HCCCLM3, scr-HCCCLM3, or si-HCCCLM3 cells were s.c. injected into nude mice, respectively. After 28 days, the xenografts were removed, cut into slices, and then implanted into the livers of three nude mice groups with eight animals per group. At the 35th day, we found that the average size of xenografts in si-HCCCLM3 group was dramatically smaller than that of the HCCCLM3 group. However, no
difference was observed between the HCCLM3 and scr-HCCLM3 groups (Fig. 5).

**In vivo inhibition of cancer metastasis.** The above-mentioned tumor-grafted livers were examined after the mice were sacrificed. Three of eight mice in si-HCCLM3 group showed intrahepatic metastasis (three of eight, 38%). Metastatic ratio of intrahepatic metastasis was obviously decreased in the si-HCCLM3 group compared with the HCCLM3 group (eight of eight, 100%), as shown in Fig. 6A and Table 1. Furthermore, the pulmonary metastasis was observed in lung tissue sections of only one mouse in si-HCCLM3 group (one of eight, 13%), much less than the ratio of pulmonary metastasis in HCCLM3 group (six of eight, 75%), as shown in Fig. 6B and Table 1. No difference in the ratio of either intrahepatic metastasis or pulmonary metastasis was observed between the scr-HCCLM3 and HCCLM3 groups (Table 1).

**Discussion**

One of the hallmarks of malignant cells in solid tumors is the low pH resulting from overly extruded acid (33). The source of acidity is mainly lactic acid, the product of glycolysis, which is enhanced in many types of tumor cells including hepatocellular carcinoma cells (34–36). The low pH is optimal for cancer cells; on the contrary, it is toxic to normal cells of host because low pH can increase ECM digestion, induce apoptosis of adjacent normal cells, promote angiogenesis, and inhibit the host immune system (1).

V-ATPase, which is overly expressed in many types of metastatic cancers, benefits for their invasion and metastasis. It seems that V-ATPase is positively related to the metastatic potential (9–12). For example, the lowly metastatic breast cancer cells preferentially utilize Na+/H+ exchangers and HCO$_3^-$–based H$^+$-transporting mechanisms, whereas the highly metastatic cells take advantage of the plasma membrane V-ATPases (12). 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, 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 (cathepsin B, D, etc.; refs. 13–15) and MMPs (MMP-2, MMP-9, MMP-3, etc.; refs. 16, 17).

Another mechanism that the active V-ATPase could facilitate invasion and metastasis may be through its influence on cellular motility and migration (37–39). In the activated osteoclasts, 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. From the viewpoint that the invasive phenotype of malignant cells is somehow similar to the activated osteoclasts, the concentration of V-ATPase may probably occur in the cell surface of an invading cancer cell, resulting in the "aggressive" protruding edge. Moreover, V-ATPase maintains acidic luminal pH, necessary for the ligand-receptor dissociation and recycling of membrane receptor (40, 41). When ECM is digested during invasion, some trapped factors that can regulate cellular proliferation, apoptosis, and adhesion will be released from the degraded ECM (42). 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, ATP6L itself has binding sites for papillomavirus E5 oncoprotein, platelet-derived growth factor and $\beta$-integrin, which can also regulate the proliferation and adhesion (43–45).

Based on the above fact that the increased production and extrusion of proton is an initiating and enhancing factor for carcinogenesis and invasion, we therefore attempted to down-regulate the V-ATPase activity via knockdown of ATP6L expression to examine the alteration of pH and pHe in the highly metastatic HCCLM3 cells and its effect on metastatic potential of these cells. Our results revealed that the down-regulation of V-ATPase activity by the knockdown of ATP6L expression using siRNA techniques could practically inhibit the invasion (Fig. 2B), proton extrusion (Fig. 3), and the metastasis of si-HCCLM3 cells (Fig. 6; Table 1).

**Figure 5.** Growth of si-HCCLM3 xenografts. The size of xenografts in the si-HCCLM3 group was dramatically smaller than that of HCCLM3 group ($^{*}P < 0.001$). No difference was observed between scr HCCLM3 group and HCCLM3 group. **Columns,** means ($n = 8$); **bars,** SE; **arrows,** tumor nodules at the sites of implantation.

**Figure 6.** Metastasis of si-HCCLM3 xenografts. A, intrahepatic metastasis. **Green arrows,** metastatic nodules; **yellow arrow,** the site of implantation. **B,** pulmonary metastasis. **Black arrow,** metastatic human hepatocellular carcinoma cells in the lung tissues of the mice. H&E stain.
Simultaneously, as also shown in Fig. 4, either endogenous MMP-2 expression or gelatinase activity in the supernatant of the cultured si-HCCLM3 cells was decreased. Therefore, our data showed that the inhibitory effect of intrahepatic and pulmonary metastasis on the si-HCCLM3 xenograft animal model resulted from the reduction of proton extrusion and the inhibition of gelatinase as well.

Knockdown of ATP6L expression may block some V-ATPase–regulated or ATP6L-mediated signal pathways and thereby affect the proliferation of cancer cells (Fig. 5). The retardation for the growth of si-HCCLM3 xenografts seems to be an outcome of multiple events. In addition to the knockdown of ATP6L expression affecting the V-ATPase–regulated or ATP6L-mediated pathways, the reduced blood supply to the si-HCCLM3 xenografts may be one of these events. The increased pHe may reduce the expression of some acid-induced angiogenesis-promoting factors, such as interleukin-8 and vascular endothelial growth factor (46, 47). Moreover, the si-HCCLM3 cells itself had low MMP-2 expression (Fig. 4A), a direct angiogenesis-promoting factor (48).

Taking our in vitro and in vivo data together, the inhibition of proton extrusion by knockdown of ATP6L expression can apparently suppress the growth and metastasis of cancer. Our data also show that the DNA vector–expressed siRNA could exert a stable silencing effect on the targeted gene ATP6L, a key subunit of proton pump V-ATPase. To our knowledge, this is the first report that the knockdown of ATP6L expression using DNA vector–based siRNA technology can suppress the growth and metastasis of human hepatocellular carcinoma. Our results implicate the potential for cancer treatment using V-ATPase as a candidate target.

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References


Table 1. The comparison of metastatic ratio among the HCCLM3 group, the scr-HCCLM3 group, and the si-HCCLM3 group

<table>
<thead>
<tr>
<th>Metastasis</th>
<th>HCCLM3</th>
<th>scr-HCCLM3</th>
<th>si-HCCLM3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intrahepatic</td>
<td>8/8, 100%</td>
<td>8/8, 100%</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Pulmonary</td>
<td>6/8, 75%</td>
<td>5/8, 63%</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Ratio</td>
<td>3/8, 38%</td>
<td>1/8, 13%</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

NOTE: P indicates possibility compared with HCCLM3 group using two-tailed Fisher’s exact test.
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