Original Article
Membrane type-2 matrix metalloproteinases improve the progression of renal cell cancer

Yiming Chen, Hao Lu, Ding Tao, Min Fan, Qianfeng Zhuang, Zhaoyu Xing, Zhen Chen, Xiaozhou He

Department of Urology, The Third Affiliated Hospital of Soochow University, 185 Juqian Street, Changzhou 213003, People's Republic of China

Abstract: Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes, which involved in the degradation of extracellular matrix (ECM) and basement membrane (BM), and associated with tumor invasion and metastasis. Membrane type-2 MMP (MT2-MMP) is a member of MT-MMPs subgroup, and is supposed to be an important step for cancer invasion and metastasis. However, the roles of MT2-MMP in human renal cell carcinoma (RCC) remain unknown. In present study, we identified the roles of MT2-MMP in renal cancer progression by MT2-MMP suppression and overexpression in ACHN cells, which expressed highest level of MT2-MMP and lowest level of MT1-MMP in three kinds of renal cancer cells (786-0, ACHN, OS-RC-2). We found that the expression of MMP-2 could be regulated by MT2-MMP suppression or overexpression in ACHN cells, and both adhesion and invasive activities of ACHN cells were suppressed with MT2-MMP siRNA transfection. In addition, we found that MT2-MMP could increase ACHN cell proliferation, and inhibit cell apoptosis. *In vitro* tumor growth experiment showed that MT2-MMP could increase clone formation of ACHN cells. The results indicated that MT2-MMP could promoter renal cancer cell invasion and adhesion by activating the expression of MMP2, and stimulate tumor growth of renal cancer.

Keywords: Renal cell carcinoma, MT2-MMP, adhesion, invasion, proliferation

Introduction

Renal cell carcinoma (RCC) is one of the most common cancers. In 2013, 350,000 new cases were diagnosed in worldwide, and RCC lead to more than 140,000 deaths per year [1]. The increasing use of imaging techniques in medical examination are helpful to finding renal tumor incidentally, but more than 17% of all RCC have distant metastases at the time of diagnosis [1]. The efficacy of chemotherapy in metastatic renal cell carcinoma is unsatisfactory. Recently, the systemic therapy based on immune modulators was used for treating renal cancers, but the improvement of efficacy were slightly [2, 3]. Therefore, novel therapies are required to improve the prognosis of RCC patients.

Matrix metalloproteinases (MMPs) are a family of enzymes involved in extracellular matrix (ECM) and basement membrane (BM) destruction. More evidences suggested that MMPs contributed to tumor invasion and metastasis. MMPs are frequently overexpressed in a variety of malignant tumors [4, 5]. MMPs contain more than 25 members, which can be classified into five subgroups [6]. Membrane-type MMPs (MT-MMPs) are a specific subtype, directing peri-cellular proteolysis, which is supposed to be an important step for the peri-cancerous tissue remodeling [7]. MT2-MMP (also named MMP-15) was originally isolated from a human lung cDNA library, which composed of 669 amino acids [8]. Recent studies indicated that MT2-MMP was involved in the progression of multiple human cancers [9-14]. However, the roles of MT2-MMP in human RCC remain unknown. The degradation of the BM by MMP-2 was considered as a necessary step for cancer invasion, and it was found that MT1-MMP and MT2-MMP were both involved in the activation of MMP-2 [7, 8]. Therefore, we hypothesized that MT2-MMP also mediated the progression of renal cancer by MMP2 activating.

In present study, we first examined both mRNA and protein expression levels of MT1-MMP and MT2-MMP in three kinds of renal cancer cell lines (786-0, ACHN, OS-RC-2) and human

Table 1. Primer sequences for qPCR

Gene	Primer sequence (5'-3')	Accession No.
ACTB	F: CATGTACGTTGCTATCCAGGC	NM_001101
	R: CTCCTTAATGTCACGCACGAT	
MT1-MMP	F: CATCTGTGACGGGAACTTTGA	NM_004995
	R: GGCAGTGTTGATGGACGCA	
MT2-MMP	F: AGGTCCATGCCGAGAACTG	NM_002428
	R: GTCTCTTCGTCGAGCACACC	
MMP2	F: GATACCCCTTTGACGGTAAGGA	NM_004530
	R: CCTTCTCCCAAGGTCCATAGC	

embryonic kidney cells (293T) by quantitative PCR (qPCR) and western blot. Next, we performed MT2-MMP RNA interference and overexpression in ACHN cells to study the roles of MT2-MMP for MMP2 expression and renal cancer progression.

Materials and methods

Cell culture

Human renal cancer cells 786-0 (ATCC® CRL-1932™), ACHN (ATCC® CRL-1611™) and 293T cells (ATCC® CRL-11268™) were obtained from American Type Culture Collection (ATCC, USA). OS-RC-2 cells were obtained from Chinese academy of sciences institute of cell resource center (Shanghai, China). All cells were cultured in appropriate medium (Roswell Park Memorial Institute-1640 medium (RPMI-1640; Hyclone Laboratories, USA) for 786-0 and OS-RC-2 cells, Eagle's Minimum Essential Medium (EMEM; No.30-2003, ATCC, USA) for ACHN cells, and Dulbecco's Modified Eagle's Medium (DMEM; Hyclone Laboratories, USA) for 293T cells), supplemented with 10% Fetal bovine serum (FBS; Procell, Wuhan, China), 100 IU/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified 5% CO_2 atmosphere.

Plasmid, siRNA and cell transfection

The full length coding sequence of MT2-MMP was amplified by LA Taq polymerase (TaKaRa, Dalian, China) according to the manufacturer's protocol. The primers were designed as the coding region of MT2-MMP (GenBank accession no. NM_002428), and the sequences were as following: F: 5'-CAAGTTTCCAAGGCGCGTG-3'; R: 5'-TGGGAGCCATCTCAGAACCA-3'. The purified PCR product was cloned into pEASY-T1 vector (TransGEN, Beijing, China). The fragments of MT2-MMP were inserted into the EcoRI and

XhoI sites of the pcDNA3.1 (+) expression vector (Invitrogen Life Technologies, Carlsbad, CA, USA). Inserted sequence was confirmed by sequencing (Sangon Biotech, Shanghai, China). The plasmid used in cell transfection experiment was purified by E.Z.N.A.® Endo-free Plasmid Mini Kit II (OMEGA Bio-Tek, USA).

The siRNA oligo was synthesized by Genechem (Shanghai, China). Sequences were as following: MT2-MMP siRNA (5'-GGTGAC-CGCTACTGGCTCT-3'). SiRNA negative control (NC siRNA) was ordered directly from Genechem (Shanghai, China).

For transfection, ACHN cells were cultured for 12 h in EMEM medium supplemented with 10% FBS, 100 units/mL penicillin, and 0.1 mg/mL streptomycin, and transfected using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. 48 h after transfection, cells were washed by PBS, and lysed in 1 mL TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) for RNA extraction. 72 h after transfection, cells were washed by PBS, and lysed in 1 × RIPA buffer (Beyotime Biotechnology, China) supplemented with 0.2 mM protease Inhibitor (PMSF, Cat: 36978, Thermo Scientific™, USA) for 30 min on ice for western blot assay.

qPCR

Total RNA was reverse transcribed by First Strand cDNA Synthesis Kit (TOYOBO, Dalian, China) following the manufacturer's recommended procedure. qPCR was conducted on StepOne™ (Life technologies, USA) in a 10 µL volume containing 5.0 µL of 2 × qPCR Mix of SYBR® Premix Ex Taq™ (TaKaRa, Dalian, China), 1.0 µL of each primers (**Table 1**) (2.5 µM), 1.0 µL of cDNA (approximate concentration is 100 ng), and 3 µL ddH$_2$O. Melting curves was used to confirm the specificity of each primer. The relative gene expression levels were calculated by the $2^{-\Delta\Delta CT}$ method [15], and the ACTB was used as the internal control. The sample containing six biological replicates was amplified in triplicate times.

Western blotting analysis

Total protein concentrations were determined by bicinchoninic acid (BAC; Thermo, USA). 40

Table 2. Primary antibody used for western blot

Target protein	Source organism	Producer	Cat#	Dilution method	Dilution ratio
β-Actin	Rabbit	TDY	TDY051	5% skim milk	1:10000
MT1-MMP	Rabbit	Abcam	ab51074	5% skim milk	1:2000
MT2-MMP	Rabbit	Abcam	ab135562	5% skim milk	1:500
MMP2	Rabbit	Abcam	ab92536	5% skim milk	1:2000

mg of total protein was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA), and the membranes were blocked with 5% skim milk powder (BD, USA) for 60 min at room temperature. Then, the membranes were incubated with primary antibodies (**Table 2**) overnight at 4°C. Subsequently, the membranes were incubated with second antibody (HRP-Goat anti Rabbit, 1:10000) for 30 min at room temperature. Target proteins were detected with Clarity™ Western ECL Substrate (Bio-red, USA). The optical density was analyzed by AlphaEaseFC. All experiments were conducted at least three times.

Cell invasion and adhesion assay

Cell invasiveness was determined by a Matrigel (30 μg; BD Biosciences, USA)-coated transwell chamber (5.0 μm pore size polycarbonate filter with 6.5 mm diameter; Costar, USA). ACHN cells (1.5×10^5 cells/well density) in 100 μL of serum-free medium were added to the upper chamber of the transwell, and 600 μL of medium containing 10% FBS was added to the lower chamber, and were cultured at 37°C in a CO_2 incubator. 36 h after incubation, invaded cells were fixed, stained with 0.1% crystal violet, and counted under a light microscope. Three random views were selected to count the number of cells, and the experiments were repeated three times.

To assess fibronectin cell-matrix adhesion, 96-well plates were first pre-coated with 50 μL of PBS-fibronectin 20 μg/mL (Sigma Aldrich, USA) overnight at 4°C. Before seeding cells, wells were blocked with 1% bovine serum albumin (Sigma Aldrich, USA) for 30 min. 72 h following transfection, ACHN cells were seeded in pre-treatment plate at 40,000 cells/well density, and cultured for 6 h at 37°C. Subsequently, cells were rinsed with PBS, and fixed with absolute ethanol for 10 min, and then stained with 0.1% crystal violet. Adhesive cells were counted under a light microscope. Three random views were selected to count the number of cells, and the independent experiments were repeated three times.

CCK-8 assay

The cell viability was detected by Cell Counting Kit-8 (CCK-8) (ZOMANBIO, Beijing, China). ACHN cells were cultured in 96-well plates for 24 h before transfection, normal saline (NS) and cell culture mediums served as the control, and three wells were prepared for each group. After transfection, 10 μL of CCK-8 solution was added to each well, and the 96-well plate was continuously incubated at 37°C for 1 h, then the OD value for each well was read at 450 nm wave length to determine the cell viability on a microplate reader (Multiskan, Thermo, USA). The assay was repeated three times.

Flow cytometric cell cycle and cell apoptosis analysis

After fixation, cells were stained with propidium iodide (PI) solution (50 μg/mL PI and 100 μg/mL RNaseA in PBS) in dark, and then subjected to cell cycle analysis. The extent of cell apoptosis was measured using Annexin V/PI double staining. 300 μL of binding buffer was used to resuspension, and 5 μL of Annexin V-FITC was added to the cell suspension for 10 min in the dark, and then 5 μL of PI was added to the cell suspension for 5 min in the dark. The cell cycle and apoptosis were analyzed with a FACS Calibur flow cytometer (BD, USA).

Plate clone formation assay

In clone formation assay, ACHN cells were plated at a density of 1000 cells/well in 24-well plates (Corning, USA), which added agarose previously. Cells were cultured for two weeks, and stained with 0.1% crystal violet. Clone formations were observed under a light microscope. Three random views were selected to count the number of colony and the independent experiments were repeated three times.

Statistical analysis

All experiments were repeated three times, and analyzed with Excel software (Microsoft Cor-
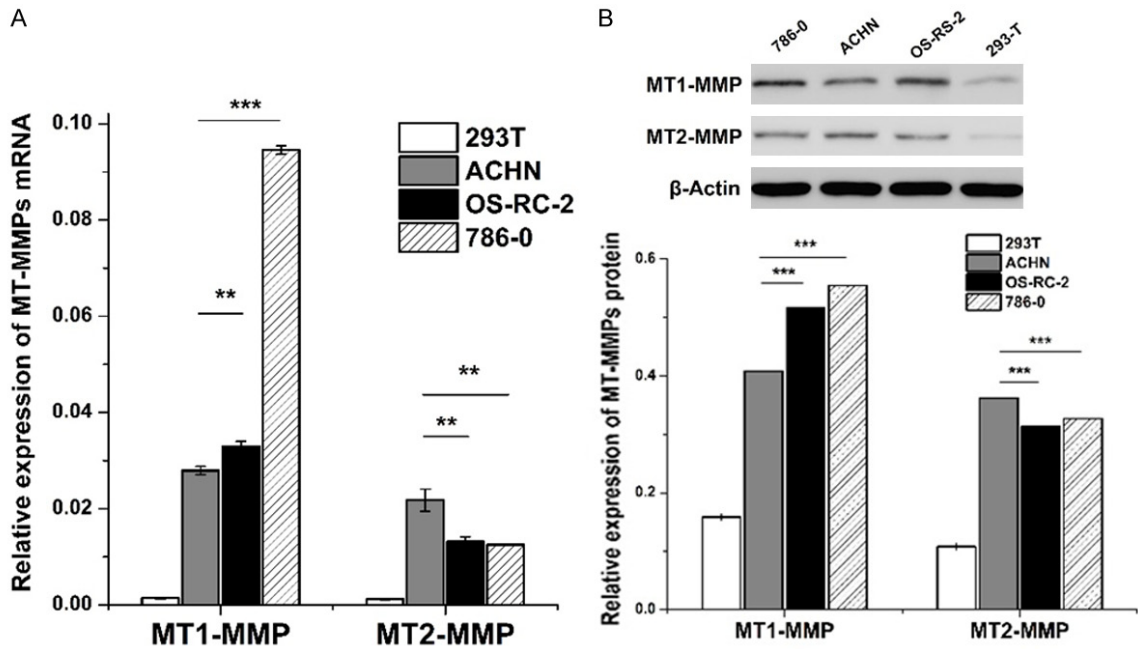
Figure 1. Expression of MT1-MMP and MT2-MMP in three kinds of renal cancer cells. A. Expression of MT1-MMP and MT2-MMP mRNA in 786-0, ACHN, OS-RC-2 and 293T cells tested by qPCR. Each bar represents the mean ± SD, n=3, **P<0.01, ***P<0.001 (t-test). B. Expression of MT1-MMP and MT2-MMP protein in 786-0, ACHN, OS-RC-2 and 293T cells tested by western blot assay. Each bar represents the mean ± SD, n=3, ***P<0.001 (t-test).
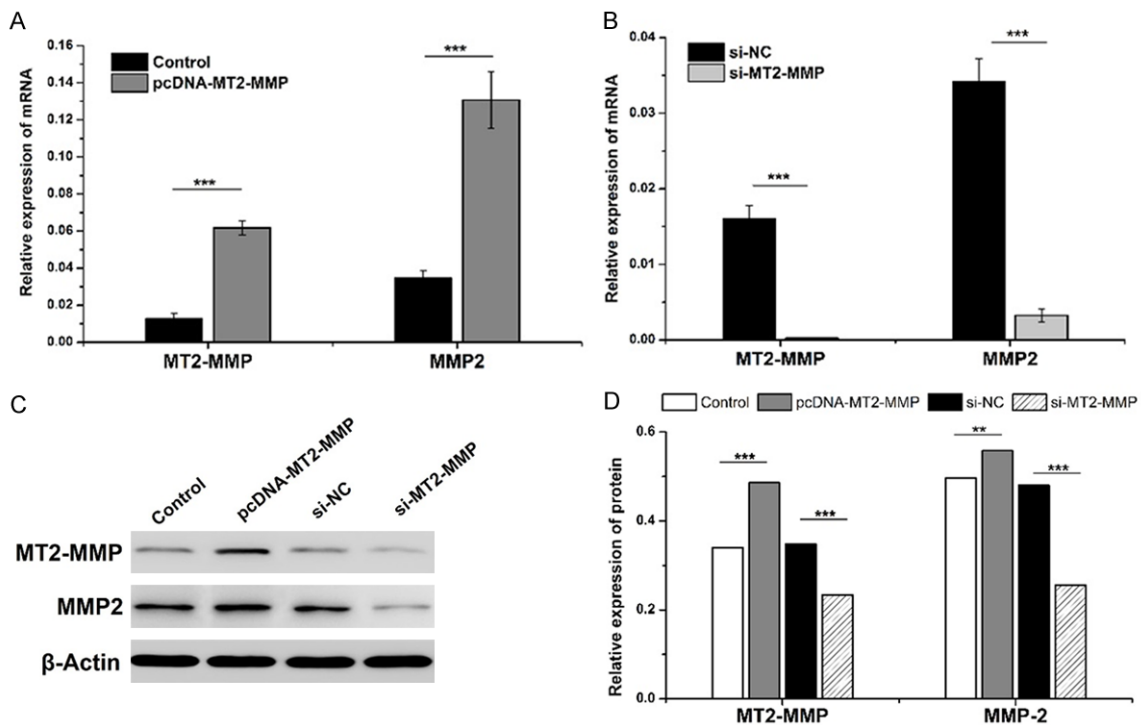

Figure 2. Expression of MT2-MMP and MMP2 by MT2-MMP overexpression or suppression. A. Expression of MT2-MMP and MMP2 mRNA were upregulated in ACHN cells transfected with pcDNA-MT2-MMP by qPCR. Each bar represents the mean ± SD, n=3, ***P<0.001 (t-test). B. Expression of MT2-MMP and MMP2 mRNA were suppressed in ACHN cells transfected with si-MT2-MMP by qPCR. Each bar represents the mean ± SD, n=3, ***P<0.001 (t-test). C, D. Expression of MT2-MMP and MMP2 protein were upregulated or suppressed in ACHN cells transfected with pcDNA-MT2-MMP or si-MT2-MMP by western blot assay, respectively. Each bar represents the mean ± SD, n=3, **P<0.01, ***P<0.001 (t-test).
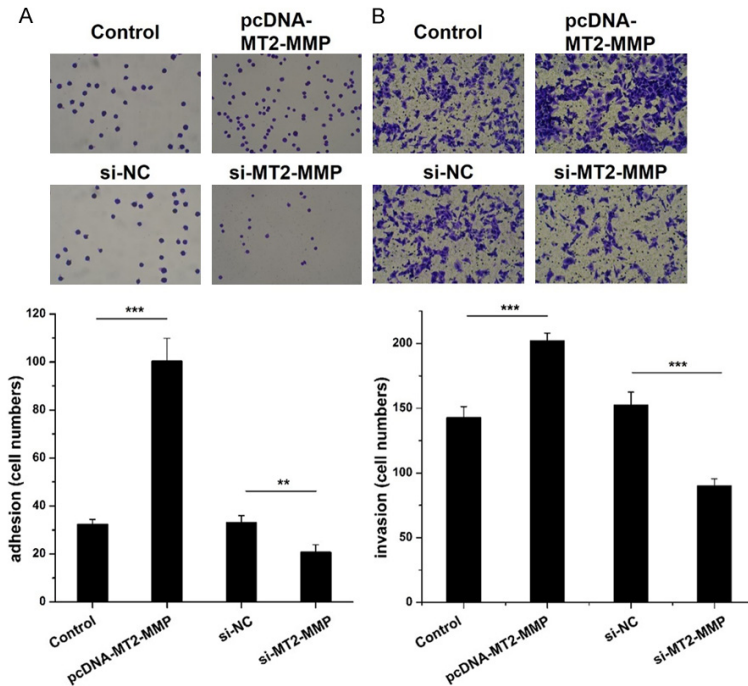
Figure 3. Effect of MT2-MMP overexpression or suppression on adhesion and invasive activity of ACHN cells. A. The adhesion activity of ACHN cells transfected with control plasmid, pcDNA-MT2-MMP, si-NC, or si-MT2-MMP were tested by cell adhesion assay. The number of adhered cells in three random fields was counted under a light microscope. Each bar represents the mean ± SD, n=3, **P<0.01, ***P<0.001 (t-test). B. The invasive activity of ACHN cells transfected with control plasmid, pcDNA-MT2-MMP, si-NC, or si-MT2-MMP were tested by transwell assay. The number of invaded cells in three random fields was counted under a light microscope. Each bar represents the mean ± SD, n=3, ***P<0.001 (t-test).

poration, USA). Statistical analyses were performed using T-test (P<0.05). Data are presented as mean ± SD.

Results

Expression of MT1-MMP and MT2-MMP in renal cancer cells

We first identified the expression of MT1-MMP and MT2-MMP in three kinds of renal cancer cell (786-0, ACHN and OS-RC-2) by qPCR and western blot. The qPCR and western blot results showed that the expression of both MT1-MMP and MT2-MMP were notably higher in renal cancer cells than that in 293T cells, and the expression level was different among these cells (**Figure 1**). ACHN cells expressed highest level of MT2-MMP and lowest level of MT1-MMP in the three kinds of cells. To assess the roles of MT2-MMP, we chose ACHN cells for further investigation.

MT2-MMP active the expression of MMP2 in ACHN cells

To observe the relationship between MT2-MMP and MM-P2, we preformed MT2-MMP suppression and overexpression in ACHN cells, respectively. Compared to control cells, the mRNA and protein expression of MT2-MMP and MMP2 were significantly increased by MT2-MMP overexpression (**Figure 2A** and **2C**). Likewise, expression level of MT2-MMP and MMP2 were markedly suppressed in ACHN cells transfected with MT2-MMP siRNA, compared with those expression in control cells transfected with negative control siRNA (si-NC) (**Figure 2B** and **2C**). The results indicated that MT2-MMP could active MMP2 expression in renal cancer cells.

MT2-MMP enhanced cell adhesion and invasive activity of ACHN cells

The cell adhesion and transwell assay were performed to evaluate the effect of MT2-MMP on cell adhesion and invasive activity of ACHN cells. As expected, both adhesion and invasive activities of ACHN cells were suppressed with MT2-MMP siRNA transfection (**Figure 3**). The effect of MT2-MMP overexpression was opposite to that of MT2-MMP knockdown.

MT2-MMP increased cell proliferation, and inhibited cell apoptosis

To evaluate the effect of MT2-MMP on cell growth, we tested cell proliferation, cell cycle and apoptosis of ACHN cells with MT2-MMP suppression or overexpression. In CCK-8 assays, proliferation of ACHN cells was significantly suppressed for 72 h treatment of MT2-MMP siRNA (**Figure 4A**). In contrast, cell proliferation was notably increased by MT2-MMP overexpression (**Figure 4A**). We further used flow cytometry to investigate the influence of MT2-MMP on cell cycle and apoptosis. Results
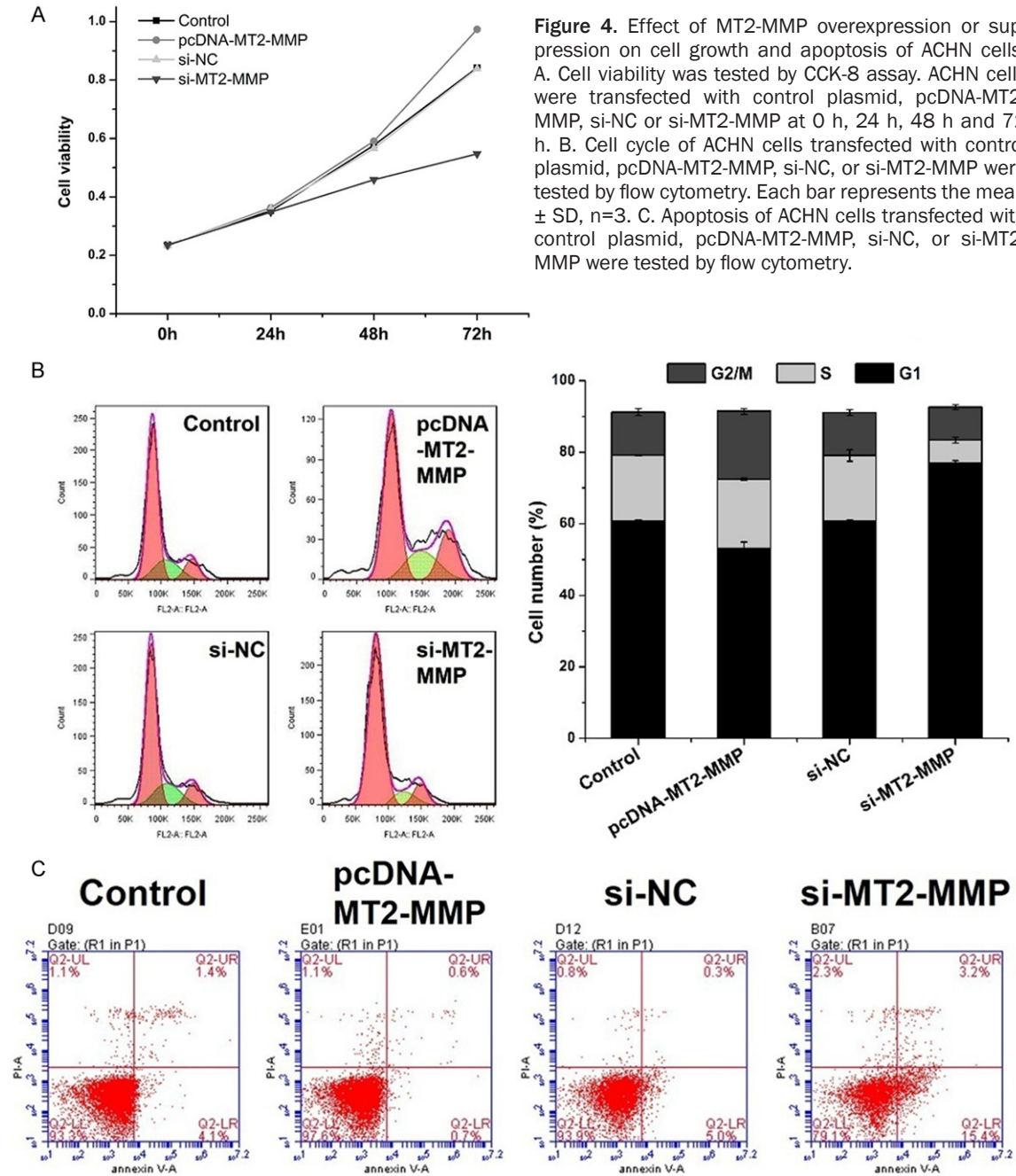
Figure 4. Effect of MT2-MMP overexpression or suppression on cell growth and apoptosis of ACHN cells. A. Cell viability was tested by CCK-8 assay. ACHN cells were transfected with control plasmid, pcDNA-MT2-MMP, si-NC or si-MT2-MMP at 0 h, 24 h, 48 h and 72 h. B. Cell cycle of ACHN cells transfected with control plasmid, pcDNA-MT2-MMP, si-NC, or si-MT2-MMP were tested by flow cytometry. Each bar represents the mean ± SD, n=3. C. Apoptosis of ACHN cells transfected with control plasmid, pcDNA-MT2-MMP, si-NC, or si-MT2-MMP were tested by flow cytometry.

showed that MT2-MMP siRNA transfection significantly declined the proportion of cells in phase S and G2/M (**Figure 4B**), and increased the ratio of apoptosis cells (**Figure 4C**). The effect of MT2-MMP overexpression was opposite to that of MT2-MMP suppression in cell cycle and apoptosis.

Effect of MT2-MMP suppression or overexpression on in vitro tumor growth of ACHN cells

Sphere and clone formation assay was used to observe the effect of MT2-MMP on the ability of tumor formation of ACHN cells *in vitro*. Compared to control cells, the number of clone formation was significantly reduced in ACHN cells transfected with MT2-MMP siRNA (**Figure 5**). The number of clone formation was increased by MT2-MMP overexpression (**Figure 5**).

Discussion

The ability of cancer cell to break down ECM, which surround all epithelial cells, was neces-
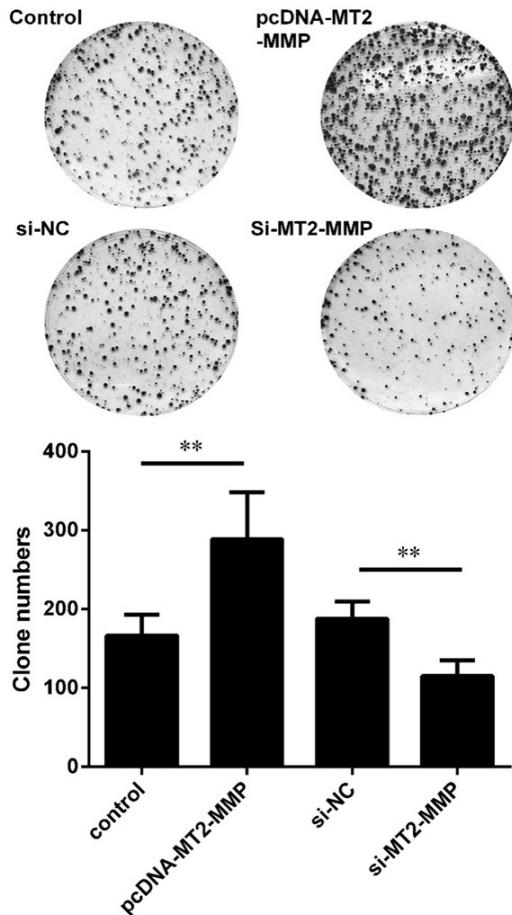
Figure 5. Effect of MT2-MMP overexpression or suppression on *in vitro* tumor growth of ACHN cells. The ability of tumorigenesis of ACHN cells transfected with transfected with control plasmid, pcDNA-MT2-MMP, si-NC, or si-MT2-MMP were tested by sphere and clone formation assay. The number of clones in three random fields was counted under a light microscope. Each bar represents the mean ± SD, n=3, **P<0.01 (t-test).

sary for cancer invasion and metastasis [16, 17]. MMPs are enzymes to degrade matrix proteins, which have been reported to play important roles in cancer progress. Among many MMPs family members, MT-MMPs have been implicated as important step to break down the pericellular matrices [7]. MT1-MMP was the first discovered MT-MMPs [18], and it was supposed to be important function to induce cell invasion and cell proliferation of cancer cells [19, 20]. Although MT2-MMP was considered to play similar roles as MT1-MMP in cancer cells [21], the roles of MT2-MMP remain to be elucidated. In present study, we explored the roles of MT2-MMP in renal cancer progression.

Recent studies suggested that MT2-MMP played a significant role in tumor progression, independent of MT1-MMP, and the TMK-1 cells, which expressed no MT1-MMP but high level of MT2-MMP, still have the ability of invasiveness and proliferation in 3D culture condition [22, 23]. In this study, we also found that the expression levels of MT1-MMP and MT2-MMP were different among three kinds of renal cancer cells (786-0, ACHN and OS-RC-2). Among these kinds of cells, ACHN cells showed the highest expression level of MT2-MMP, and the lowest expression level of MT1-MMP. Therefore, we chose ACHN cells to further investigation.

Like MT1-MMP, MT2-MMP was found to be an activator of proMMP-2 on cell surface [7, 8]. MMP2, a member of MMPs, is a proteolytic enzyme of type IV collagen, which is a major and specific BM matrix protein [24]. The degradation of BM by MMP-2 was a necessary step for cancer invasion. The expression of MMP2 was significantly increased in many types of cancer [25], and down-regulation of MMP2 could suppress cancer metastasis, invasion and adhesion [26-28]. Here, we identified that the expression of MMP-2 could regulate by MT2-MMP suppression or overexpression in ACHN cells. Furthermore, both adhesion and invasive activities of ACHN cells were suppressed with MT2-MMP siRNA transfection. The results suggested that MT2-MMP might contribute to renal cancer cell invasion and adhesion by the activation of MMP2.

Except for enhancing the ability of cell invasion and adhesion, MT2-MMP was identified as an anti-apoptotic factor in cancer cells [29]. Therefore, we presumed that MT2-MMP could influence cancer cell growth. The results of both CCK-8 assays and cell cycle assay suggested that MT2-MMP could increase ACHN cell proliferation. Furthermore, the proportion of apoptosis cells were increased by MT2-MMP suppression. In our clone formation assay, the clone formations were declined by MT2-MMP suppression in ACHN cells. These results implied that MT2-MMP had ability to increase tumor growth.

In conclusion, our results demonstrated that MT2-MMP might improve the renal cancer progression by activating MMP2 for promoting cell invasion and adhesion. The functions of MT2-

MMP presented here bring a new light for developing more effective strategies for targeted therapy of renal cell cancer. However, the underlying mechanisms of MT2-MMP during cancer progression need further investigation.

Disclosure of conflict of interest

None.

Address correspondence to: Xiaozhou He, Department of Urology, The Third Affiliated Hospital of Soochow University, 185 Juqian Street, Changzhou 213003, People's Republic of China. E-mail: hexiaozhoucz@126.com

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