# Expression of matrix metalloproteinase-26 promotes human glioma U251 cell invasion *in vitro* and *in vivo*

YIPING DENG[1], WEI LI[1], YILEI LI[1], HONGFA YANG[2], HUA XU[1], SHANSHAN LIANG[1], LIHONG ZHANG[1] and YULIN LI[1]

[1]The Key Laboratory of Pathobiology, Ministry of Education, Norman Bethune College of Medicine, [2]The First Clinical Hospital, Jilin University, Jilin, Changchun 130021, P.R. China

Received June 10, 2009; Accepted July 28, 2009

DOI: 10.3892/or_00000607

**Abstract.** MMP-26 is a novel member of the MMP family and is widely expressed in cancer cells of epithelial origin. Published research shows that MMP-26 contributes to tumor development and to the restoration of tissue injury. In this study, in order to identify the functions of MMP-26 that contribute to the biological phenotype and behavior of non-epithelial human glioma U251cells, we established an MMP-26 overexpressing tumor cell model using gene transfection. We then used these cells to investigate the role of MMP-26 in tumor progression. Adherence and spreading assay, wound healing assay, Boyden chamber invasion assay, and *in vivo* tumorigenicity assay were performed to analyze the invasion ability of MMP-26 transfected U251 cells. Microvessel density analysis and tumor cell induced angiogenesis assay were employed to detect the function of MMP-26 in angiogenesis. Results showed that the spreading cell ratio of MMP-26 transfected cells was significantly higher than parental U251 cells. The relative migration distance of MMP-26 transfected cells on Matrigel was significantly higher than that of parental U251 cells. The Boyden chamber assay showed that MMP-26 could significantly enhance the ability of U251 cells to invade through Matrigel. MMP-26 could also enhance the local invasion ability of U251 cells *in vivo*. There was a significant increase of the microvessel density of tumor tissue derived from MMP-26 transfected U251 cells. The vessel numbe induced by MMP-26 transfected U251 cells in nude mice was also significantly higher than that induced by parental U251 cells. In conclusion, we successfully established an MMP-26 overexpressing cell model and confirmed that MMP-26 contributed to U251 cell invasion and migration *in vitro*. We also demonstrated that MMP-26 plays an important role in local invasion, and angiogenesis.

*Correspondence to*: Dr Yulin Li and Dr Wei Li, The Key Laboratory of Pathobiology, Ministry of Education, Norman Bethune College of Medicine, Jilin University, 126 Xinmin Street, Changchun 130021, P.R. China
E-mail: ylli@mail.jlu.edu.cn
E-mail: lee5019@hotmail.com

*Key words:* invasion, MMPs, MMP-26, angiogenesis, glioma

## Introduction

Matrix metalloproteinases (MMPs) are a large group of zinc-dependent proteolytic enzymes which contribute to extracellular matrix (ECM) degradation. MMPs are thought to play essential roles in physiological and pathological processes of tissue remodeling and tumor progression especially in tumor invasion and angiogenesis (1-4). Many MMPs can promote angiogenesis which is a necessary process for tumor progression (5).

MMP-26 (Matrilysin-2, endometase) is a novel MMP, which was originally cloned from fetal tissue (6), placental cDNA (7), and an endometrial tumor (8,9). It has structural features that are distinct from other MMPs but is closely related to MMP-7 (Matrilysin). It is the shortest MMP identified to date, containing a signal sequence, prodomain, and a catalytic domain, but not a hinge region or hemopexin domain (7). These minimal organized structural domains contribute to its secretion, proenzyme activation, and activity (7). There is a highly conserved PRCGXXD cysteine-switch motif in the prodomain of all known MMPs, which is involved in the activation of latent enzymes. However, this conserved cysteine-switch is replaced by the unique PH[81]CGVPD sequence in MMP-26 (10). This special structural feature is to some extent a reflection of its unconventional enzyme activation mechanism, transcriptional regulation mechanism, and unusual function in physiological and pathological processes. MMP-26 can degrade most of the ECM components (11-13). MMP-26 can also activate proMMP-9 through cleavage of Ala93-Met94 site of the prepro-enzyme (14). These features suggest potential functions for MMP-26 in tumor progression and angiogenesis.

MMP-26 shows unique but complex expression patterns in normal and abnormal tissue. MMP-26 can be detected in a limited number of normal adult tissues, such as the uterus, placenta and kidney (9). However, MMP-26 is highly expressed in the mid-cycle of the endometrium and in wound repair process (15-18). MMP-26 is also widely expressed in tumor cells that are derived from epithelia such as the breast, lung, prostate, and endometrial carcinomas and also some of their corresponding cell lines (9). The elevated expression of MMP-26 is correlated with myometrial invasion of endometrial carcinoma (17,19). MMP-26 also contributes to the local depth of invasion in breast carcinoma, prostate carci-

noma and esophageal squamous cell carcinoma (14,20-26). The expression of MMP-26 seems to be located in the invading cells. MMP-26 transfected tumor cell lines also show enhanced invasive ability *in vitro*. It seems that MMP-26 contributes to the invasion process but not the malignant grade of cancers.

These data prompted us to hypothesize that MMP-26 contributes to the initiation of the invasion process of tumor cells and to angiogenesis. In this study, we screened epithelial and non-epithelial cell lines for expression of MMP-26. An MMP-26 overexpressing cell line was established by transfecting the proMMP-26 gene into MMP-26 negative U251 glioma cells. *In vitro* assays were conducted to evaluate spreading and adherence of MMP-26 transfected cells on extracellular matrix components, migration and invasion in a wound healing assay and in Boyden chamber analysis. Invasion and angiogenesis were analyzed *in vivo* in a subcutaneously transplanted tumor formed by MMP-26 transfected cells in nude mice. The results show that MMP-26 can enhance the local invasion of U251 cells *in vivo* and *in vitro*. MMP-26 may promote angiogenesis through the invasion process.

## Materials and methods

*Cell culture*. U251, MCF-7, PC-3, PC-3M, and A549 cells which respectively are an established human glioma cell line (27), a human breast carcinoma cell line (28), a human prostate carcinoma cell line (29), a highly metastatic human prostate carcinoma cell line (30) and a lung carcinoma cell line (31), were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% $CO_2$ at 37˚C.

*Transfection and isolation of MMP-26 stably transfected clones*. U251 cells were transfected with a proMMP-26 cDNA encoding vector (MMP-26-pcDNA3.1-neo, kindly prepared by Dr Alex Strongin of the Burnham Institute, La Jolla, CA) using Lipofectamine 2000 (Invitrogen, USA) as described by the manufacturer. Geneticin (G418; Merck, Germany) was employed at a concentration of 400 $\mu$g/ml to select the MMP-26 stable transfectants. Single clones of MMP-26 transfected cells were screened on the basis of MMP-26 expression and used to perform the subsequent biological tests *in vitro* and *in vivo*. U251 cells, transfected with the pcDNA3.1 vector, using the same protocol, served as a vector control along with parental U251 cells.

*Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis*. RNA was isolated from the experimental cells and transplanted tumor tissue by TRIzol (Invitrogen) according to the manufacturer's protocol. RNA (2 $\mu$g) was transcribed into cDNA by Avian Myeloblastosis Virus (AMV) reverse transcriptase and Olignucleotide (T) 18 primer. cDNA (2 $\mu$l) was added into the PCR system (Takara, Dalian, China) for 30 cycles at an annealing temperature of 55˚C. MMP-26 and ß-actin were amplified using the following primer pairs: 5'-TGACATGCAGATGCATGCTCTGC-3' (MMP-26 forward), 5'-CACTTACTGGTATCACGACCCTAG-3' (MMP-26 reverse); 5'-TGGAATCCTGTGGCATCCATGA AAC-3' (ß-actin forward), 5'-TAAAACGCAGCTCAGTA ACAGTCCG-3' (ß-actin reverse). Amplification of ß-actin was used to estimate the efficiency of cDNA synthesis and equal loading. The amplified products were fractionated on 1% agarose gel electrophoresis. The gel was stained with ethidium bromide and photographed under a gel photography system (Kodak, Japan). The products were quantified by analyzing the density of bands on the gel. Results are expressed as the mean (± SD) of the band optical density.

*Immunocytochemistry and immunohistochemistry*. Cells were plated on Fibronectin-precoated chamber glass slides and grown until confluent. The cells were then fixed in 4% paraformaldehyde for 30 min at room temperature and permeated with 0.1% Triton X-100 for 15 min. The transplanted tumor tissues were fixed in 10% paraffin and sectioned at 4 $\mu$m thickness. These sections were dewaxed with xylene and rehydrated in ethanol. Cell slides and tissue slides were blocked with normal goat serum and further incubated with specific rabbit anti-human MMP-26 antibody (12) (kindly prepared by Dr Alex Strongin of the Burnham Institute, La Jolla, CA) (1:1000 dilution) or rabbit Anti-human Factor VIII-related antigen polyclonal antibody (Zhongshan Goldenbridge Biotechnology, Beijing, China; 1:100 dilution) overnight at 4˚C. Sections were subsequently incubated with a biotinylated goat anti-rabbit IgG (SP kit; Maixin, China) for 2 h and then reacted with avidin-biotin complex (SP kit) and developed with diaminobenzidine tetrahydrochloride (DAB; Maixin, China), which produces a brown reaction product. Slides were mounted after staining the cell nuclei with hematoxylin and studied under a microscope (Olympus, Japan).

*Adherence and spreading assay*. A single cell suspension in serum-free medium was prepared by trypsinization of the transfected cell lines and controls. Cells ($1x10^4$) were plated on the fibronectin (BD Bioscience) and Matrigel (BD Bioscience) precoated 96-well culture plates. Each group was examined in 8 replicates. Cells were incubated for 2 h and washed with phosphate-buffered saline (PBS). The number of adherent and spread cells was counted by microscopy at a magnification of x200. Twenty visual fields were employed to quantify the cell number for each experimental group. Results were expressed as mean (± SD) of cell number per visual field.

*Wound healing assay*. Cells were detached and prepared in a single cell suspension. Cells ($1x10^4$) were seeded onto a Matrigel-precoated 24-well culture plate and then cultured to at least 95% confluence. Monolayer cells were washed three times with phosphate-buffered saline. A cell wound was created with a 10-$\mu$l micropipette tip and photographed at a magnification of x100. Monolayer cells with cell wounds were cultured in serum-free medium for another 24 h and then photographed at the same position. The width of the cell wounds was calculated using Adobe Photoshop software 9.0. The relative migration distance was calculated by the following formula: the relative migration distance (%) = 100 (A - B)/A, where A the width of cell wounds before incubation, B, the width of cell wounds after incubation. Results are expressed as the mean (± SD).

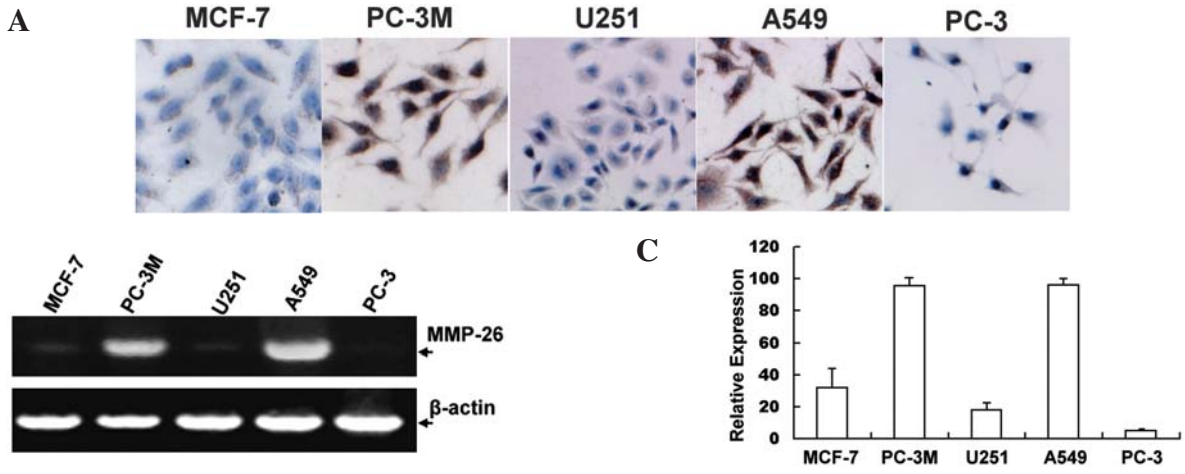



Figure 1. Expression of MMP-26 in different human tumor cell lines including breast carcinoma MCF-7, prostate carcinoma PC-3M and PC-3, glioma U251 and lung adenocarcinoma A549. Immunocytochemistry (A, original magnification, x200) and reverse transcriptase-PCR (B) were employed to detect MMP-26 expression by polyclonal antibody and designed primers of MMP-26, respectively. (A) The brown color in the cytoplasm indicates the positive expression of MMP-26. (B) Amplification of ß-actin was used to ascertain RNA integrity and equal loading. The reverse transcriptase-PCR results were quantified (C) by measuring the optical density. High expression of MMP-26 was detected in PC-3M and A549 cells whereas low in MCF-7 and barely detectable in U251 and PC-3 cells.

*Boyden chamber invasion assays*. Cells were harvested when grown to confluence and stained by 500 $\mu$g/ml Rodamine 123 (Invitrogen) for 15 min at 37˚C, and then resuspended in Eagle's MEM (Invitrogen) without fetal bovine serum. Boyden chambers which contain polycarbonate filters with 8-$\mu$m pores were precoated with 60 $\mu$l Matrigel (1:3 dilution) and incubated for 30 min at 37˚C. Cells ($1x10^4$) were seeded into each of the chambers which were then placed into a 24-well culture plate. Conditioned medium (100 $\mu$l) (24 h) from 3T3 cells was added into the lower chamber to create a chemotactic gradient. After incubation at 37˚C with 5% $CO_2$ for 3 h, the cells remaining inside the upper compartment of the chamber were carefully removed. The filters were photographed under a laser confocal microscope (Olympus, Japan). Cells which had migrated onto the lower surface of the membrane were counted under the microscope at a magnification of x100. Twenty visual fields were counted on each filter. Results are expressed as mean (± SD) of the number of cells per visual field.

*In vivo tumorigenicity assay*. Cells were harvested by trypsinization and resuspended in serum-free medium at a concentration of $1x10^7$ cells/ml. Medium (0.2 ml), containing $2x10^6$ cells were injected subcutaneously into 6- to 8-week-old female nude mice (nu/nu; Lianhelihua Co., Beijing). The animals were bred in the Center of Experimental Animals of Jilin University under standard pathogen-free conditions. Care and housing were under the supervision of authorized investigators of the local Ethics Committee for Animal Research at Jilin University and in accordance with the international standards for animal welfare. Tumor volumes were estimated every 3 days by measuring the long and short diameter of the tumor. After 42 days, these animals were sacrificed by cervical dislocation. Half of each tumor and all main organs, including liver, spleen, pancreas and heart were harvested and fixed in 10% formalin, dehydrated, embedded in paraffin, and sectioned at 4 $\mu$m thickness for histological analyses and immunohistochemistry. Sections were stained with hematoxylin-eosin for histological analyses by light microscopy and immunohistochemistry. The other half of each tumor was frozen in liquid nitrogen for subsequent RNA extraction.

*Microvessel density analysis*. Sections of the transplanted tumors were dewaxed with xylene and rehydrated in ethanol. Microvessels were stained with primary rabbit Anti-human Factor VIII-related antigen polyclonal antibody as described above. The stained vessels were observed under light microscopy at a magnification of x100. Ten visual fields of each tumor were employed to quantify the vessel number. Results are expressed as mean (± SD) of vessel number per visual field.

*Tumor cell induced angiogenesis assay*. Cells were harvested by trypsinization and resuspended in serum-free medium at a concentration of $1x10^7$ cells/ml. Medium (0.1 ml), containing $1x10^6$ cells, was injected subcutaneously into 6- to- 8-week old female nude mice (nu/nu; Lianhelihua). Nude mice were sacrificed at day 4 after the injection. The injection site was photographed under the dissecting microscope at x7 magnification. Twenty visual fields of each nude mouse were employed to quantify the vessel number. Results are expressed as mean (± SD) of vessel number per visual field.

*Statistical analysis*. Differences between the experimental conditions were evaluated using the one-way anova test and Fisher's exact test through the SPSS software according to the date format. P-values $<0.05$ were considered significant.

## Results

*Expression of MMP-26 in different tumor cell lines*. In order to seek evidence for MMP-26 expression in tumor cell lines of epithelial and non-epithelial origin, we used reverse transcriptase PCR and immunocytochemistry to examine MMP-26 expression in epithelial origin cell lines including breast carcinoma MCF-7, prostate carcinoma PC-3 and

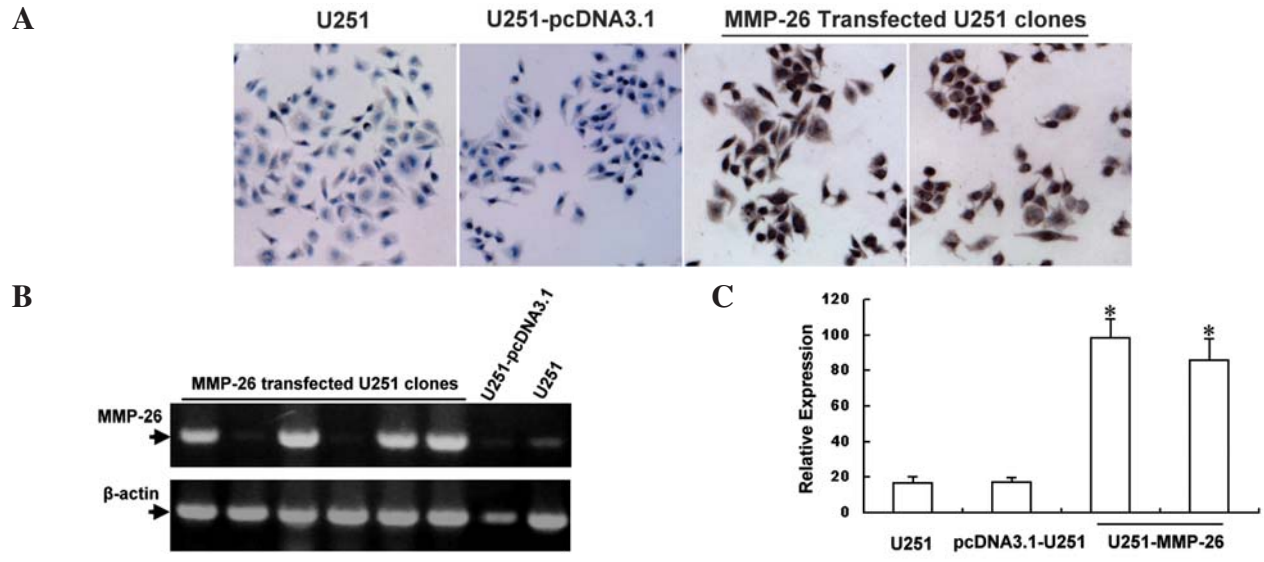



Figure 2. Expression of MMP-26 in MMP-26 transfected U251 cells. proMMP-26 encoding cDNA was transfected into U251 cells using Lipofectamine 2000. Single cell clones were collected to identify the overexpression of MMP-26 employing immunocytochemistry (A, original magnification, x150) and reverse transcriptase-PCR (B). (A) MMP-26 transfected cell clones showed high expression of MMP-26 in the cytoplasm. (B) Amplification of ß-actin was used to ascertain RNA integrity and equal loading. (C) Optical densities show that the expression of MMP-26 in the transfected cell clones was significantly higher than in the non-transfected U251 cells and pcDNA-3.1 vector transfected cells. *Indicates statistically significant difference in expression of MMP-26 in transfected cells compared with expression in non-transfected U251 cells, $p<0.001$.

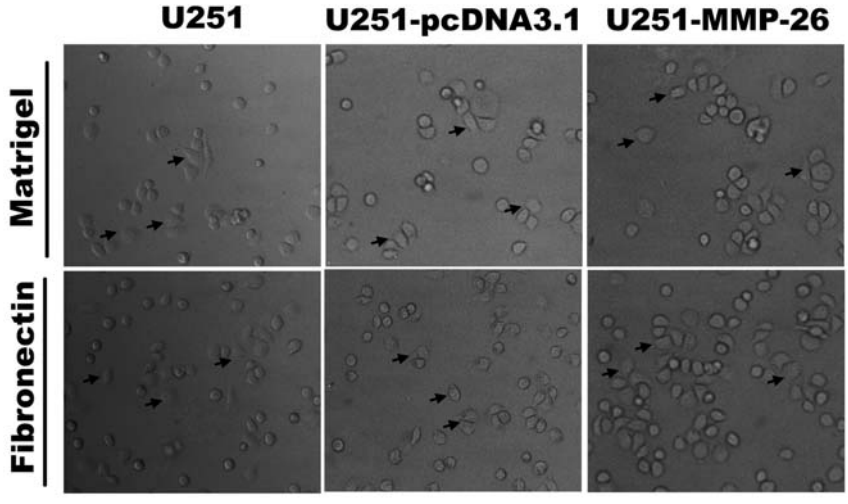
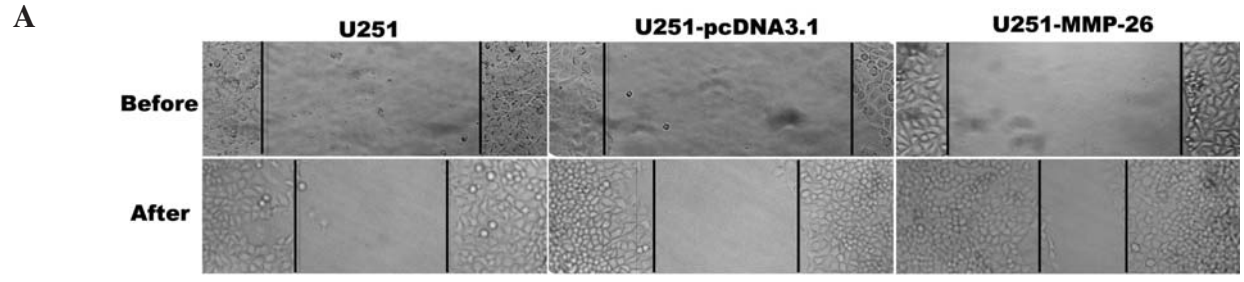



Figure 3. Adherence and spreading of cells on Matrigel (upper panel) and Fibronectin (lower panel). Cells ($1x10^4$) of each group were seeded into 96-well plates precoated with Matrigel or Fibronectin. Cells were washed three times and photographed after incubation for 2 h. Photographed cells were adherent to the Matrigel or Fibronectin. Arrow indicates the spreading cells.

Table I. Adherence and spreading of U251-MMP-26 cells on Matrigel and Fibronectin.

| | Matrigel | | Fibronectin | |
|---|---|---|---|---|
| Group | Adhering cells/field | Spreading cell ratio | Adhering cells/field | Spreading cell ratio |
| U251 | 73.4±11.2 | 36.3±5.2 | 93.1±14.9 | 43.1±6.0 |
| U251-pcDNA3.1 | 78.5±9.3 | 37.1±8.6 | 92.2±12.4 | 42.3±4.7 |
| U251-MMP-26 | 77.3±13.5 | 62.7±10.5[a] | 104.8±17.9[b] | 56.1±5.6[a] |

(n=20, $\overline{X}$ ± SD). Adherent cells and spreading cells were counted under an inverted microscope at a magnification of x200. Twenty visual fields were counted. Spreading cell ratio refers to the percentage of adherent cells that showed features of spreading. [a]$p<0.001$; [b]$p<0.05$, compared with U251 group.



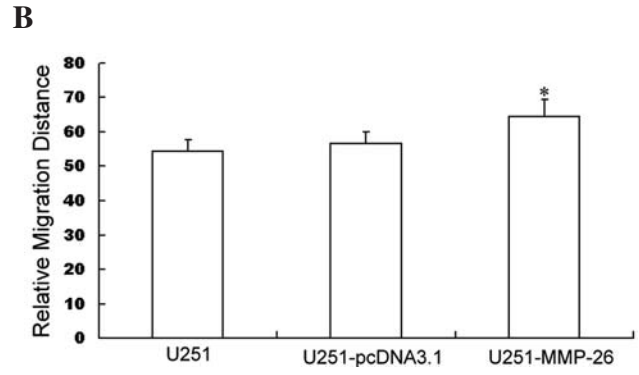




Figure 4. Wound healing analysis on Matrigel. (A) Cells ($1x10^4$) were seed into 24-well cell culture plates precoated with Matrigel and then cultured to 95% confluence. A cell wound was created with a 10 $\mu$l micropipette and photographed at a magnification of x100 (upper panel). Cells were then photograghed at the same position after 24 h (lower panel). (B) The relative migration distance was calculated using the protocol described in Materials and methods. *p<0.05, compared with U251 group.

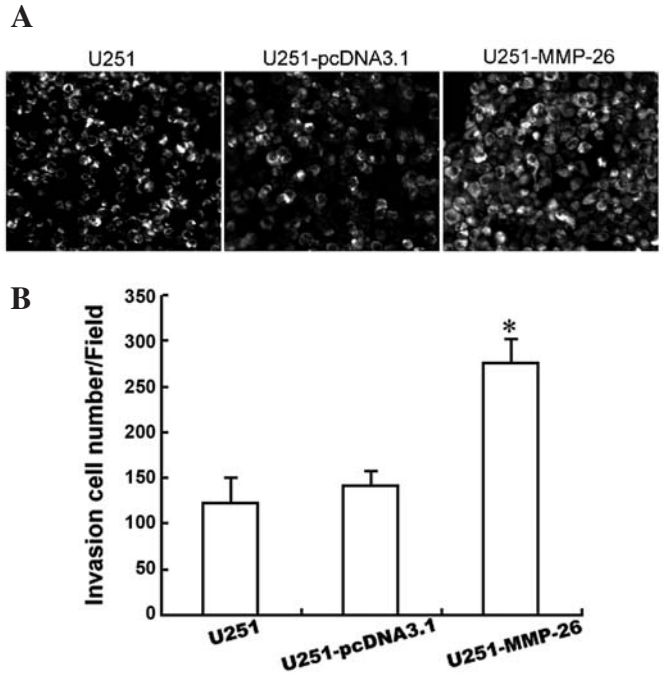



Figure 5. Invasion of U251-MMP-26 cells through Matrigel by modified Boyden chamber analysis described in Materials and methods. (A) Cells that migrated to the lower surface of the membrane were photographed with a laser confocal microscope at a magnification of x100. (B) The numbers of cells which had invaded to the lower surface of the chamber membrane. Invasive cell number was quantified by counting the invasive cells as described. Data are expressed as mean (± SD). *p<0.05, compared with U251 group.

PC-3M, and lung carcinoma A549 as well as the non-epithelial glioma U251 (Fig. 1A and B). Immunocytochemistry (Fig. 1A) and semi-quantified mRNA level of MMP-26 (Fig. 1B and C) indicated the high expression of MMP-26 in PC-3M and A549 cells whereas expression was low in MCF-7 cells and barely detectable in U251 and PC-3 cells. Consequently, U251 cell line which lacks MMP-26 expression, was chosen as the target cell line for transfection.

*Expression of MMP-26 in transfected U251 cells*. We looked for expression of MMP-26 in parental U251 cells, pcDNA3.1 vector transfected U251 cells, and MMP-26 transfected cells. Immunocytochemistry showed high expression of MMP-26 in the cytoplasm of MMP-26 transfected cell clones whereas there was minimal or no expression in parental U251 cells and vector transfected U251 cells (Fig. 2A). MMP-26 mRNA level in MMP-26 transfected cells was significantly higher ($p<0.05$) (Fig. 2B and C) than in parental U251 cells and vector transfected U251 cells through reverse transcriptase PCR analysis.

*Effects of MMP-26 on the invasive behavior of U251 cells in vitro*. The adherence and spreading assay showed that MMP-26 transfected cells, vector transfected cells, and parental cells attached to Fibronectin and Matrigel with round and spreading features after 2-h incubation (Fig. 3). There was a significantly ($p<0.05$) increased spreading cell ratio of MMP-26 transfected cells (Table I). The migration assay (Fig. 4A) showed that the relative migration distance of MMP-26 transfected cells was significantly ($p<0.05$) higher than that of vector transfected cells or parental U251 cells (Fig. 4B). MMP-26 transfected cells, vector transfected cells, and parental U251 cell invaded through Matrigel during Boyden chamber assay (Fig. 5A). MMP-26 transfected cells showed significantly ($p<0.05$) increased invasive ability through Matrigel when compared with parental U251 cells (Fig. 5B).

*Expression of MMP-26 in subcutaneously transplanted tumors*. MMP-26 expression was confirmed in transplanted tumor tissues derived from parental U251 cells, pcDNA3.1 vector transfected U251 cells, and MMP-26 transfected cells (Fig. 6). Immunohistochemistry showed strong staining of MMP-26 in tumor tissues derived from MMP-26 transfected cells whereas there was no expression in tumor tissues derived

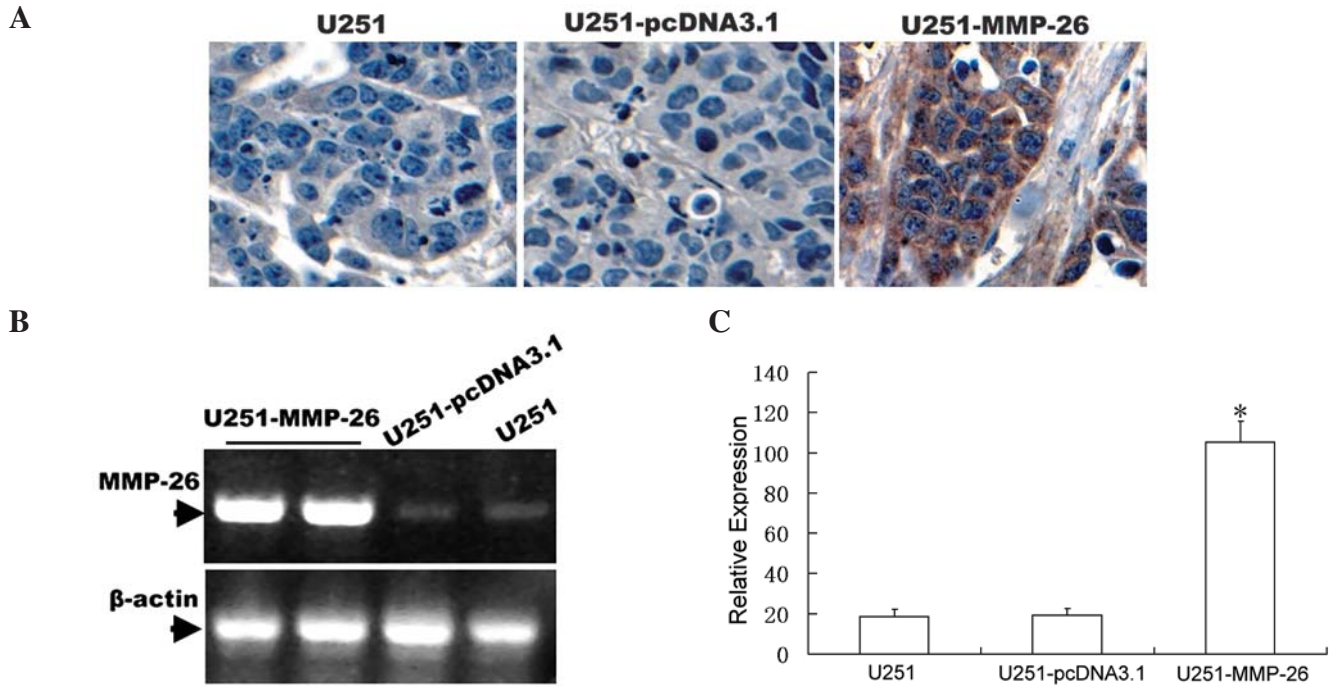



Figure 6. Expression of MMP-26 by subcutaneously transplanted tumor in nude mice. Nude mice were sacrificed 42 days after subcutaneous injection of $2x10^6$ cells. Tumor tissues were harvested. Half of each tumor was fixed in 10% formalin, dehydrated, embedded in paraffin, and sectioned at 4 $\mu$m thickness. The other half of each tumor was frozen in liquid nitrogen for RNA extraction. Expression of MMP-26 was detected using immunohistochemistry (A) and reverse transcriptase PCR (B). The brown color in the cytoplasm (A) indicates the positive expression of MMP-26. Amplification of ß-actin (B) was used to ascertain RNA integrity and equal loading. The reverse transcriptase-PCR results were quantified (C) by measuring the optical density as described. The tumors derived from MMP-26 stably transfected U251 cells show high expression of MMP-26. *Indicates statistically significant difference in expression of MMP-26 in tumors derived from U251-MMP-26 cells compared with expression in tumors derived from U251 cells, $p<0.001$.

Table II. Invasion status of subcutaneously transplanted tumor in nude mice.

| Invasion status | U251 | U251-pcDNA3.1 | U251-MMP-26 |
|---|---|---|---|
| Satellite tumor formation | 1/5 | 1/5 | 6/9 |
| Vessel invasion | 1/5 | 0/5 | 4/9 |
| Muscle invasion | 1/5 | 1/5 | 4/9 |
| Peritoneal cavity invasion | 0/5 | 0/5 | 2/9 |

Experimental nude mice were divided into three groups (5 for U251 group, 5 for U251-pcDNA3.1 group and 9 for U251-pcDNA3.1) in random. Tumor cells ($2x10^6$) in 0.2 ml serum free medium were injected into the nude mice subcutaneously. Tumor tissues and organs were harvested after 42 days. Histological analysis under light microscope through hematoxylin-eosin analysis was performed as described. Tumor cells showed different invasion status including satellite tumor formation, vessel invasion, muscle invasion and peritoneal cavity invasion.

from parental U251 cells and vector transfected U251 cells (Fig. 6A). MMP-26 mRNA level in tumor tissues derived from MMP-26 transfected cells was significantly higher ($p<0.05$) (Fig. 6C) than other tumor tissues through reverse transcriptase PCR analysis (Fig. 6B).

*Effects of MMP-26 on local tumor invasion*. Transplanted tumor tissues were harvested and sectioned. Hematoxylin-eosin staining was performed to observe the invasion status of subcutaneously transplanted tumors (Fig. 7). Tumors in both MMP-26 transfected group and control groups showed an infiltrative and lobulated growth pattern. U251-MMP-26 tumor cells were more destructive and infiltrative with distinctive undefined capsules comparing to the U251 tumor cells and U251-pcDNA3.1 tumor cells. The formation of satellite tumors was marked in six nude mice (Fig. 7A, Table II). In four nude mice, U251-MMP-26 broke through the basement membrane of blood vessels to form tumor emboli (Fig. 7B, Table II). U251-MMP-26 tumor cells in four nude mice were seen to invade muscle tissue (Fig. 7C, Table II). Intriguingly, some of U251-MMP-26 tumor cells in two nude mice penetrated into the peritoneal cavity and invaded the liver (Fig. 7D), spleen (Fig. 7E) and pancreas (Fig. 7F, Table II).

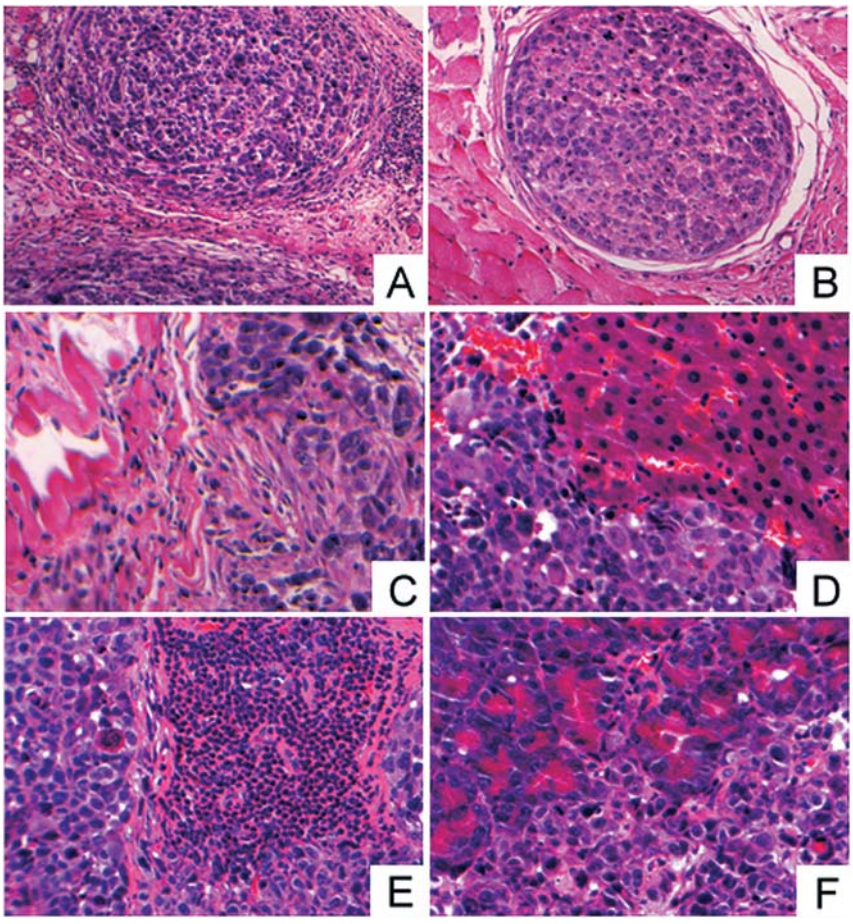



Figure 7. Invasion status of subcutaneously transplanted tumors in nude mice. Tumor tissues were harvested and sectioned as described. Hematoxylin-eosin staining was performed to observe the invasion status of subcutaneously transplanted tumors. These tumors showed infiltrative and lobulated growth pattern. Tumor nudules were surrounded by pseudocapsules. U251-MMP-26 tumor cells broke through the pseudocapsule to form satellite tumors (A) and broke through the basement membrane of blood vessels to form tumor emboli (B). U251-MMP-26 tumor cells also invaded muscle tissue (C). Intriguingly, some of U251-MMP-26 penetrated into the peritoneal cavity and invaded liver (D), spleen (E) and pancreas (F). Original magnification, (A-C), x100; (D), x200; (E-H), x400.

*Effects of MMP-26 on angiogenesis*. We examined the effects of MMP-26 on angiogenesis by assaying the microvessel density in transplanted tumor tissues (Fig. 8) and tumor cell-induced angiogenesis assay (Fig. 9). Microvessels were identified by Anti-human Factor VIII-related antigen polyclonal antibody in the microvessel density assay (Fig. 8A). The microvessel density of tumor tissues derived from MMP-26 transfected U251 cells was significantly ($p<0.05$) higher than that of tumor tissues derived from vector transfected U251 cells and parental U251 cells (Fig. 8B). In the tumor cell-induced angiogenesis assay, vessels induced by transfected MMP-26 cells in nude mice formed a vascular network (Fig. 9A). Vessels induced by transfected MMP-26 cells were significantly ($p<0.05$) higher in number than vessels induced by non-transfected U251 cells or by cells that had been transfected by the vector alone (Fig. 9B).

## Discussion

Tumor invasion and metastasis are the most distinctive characteristics of malignant neoplasms. These are complicated processes, accompanied by extracelluar matrix degradation and changes in cell surface receptor expression. Matrix metalloproteinases (MMPs) are among the most important proteolytic enzymes involved in tumor development and progression. They contribute to extracelluar matrix breakdown, tumor growth and proliferation, angiogenesis, and regulation of cell adherence and spreading. MMP-26 is a novel and structurally the simplest member of the MMP family. It is widely expressed in cancer cells of epithelial origin (9) and is correlated with the estrogen-dependent tumors including breast cancer (22,26), endometrial cancer (8) and ovarian cancer (32). In breast cancer, expression of MMP-26 in ductal carcinoma *in situ* (DCIS) was significantly higher than that in infiltrating ductal carcinoma (IDC) (22). MMP-26 can be highly expressed in prostate cancer and has been shown to promote invasion of prostate cancer cells through basement membrane by activating proMMP-9 (14). Most previous studies have focused on tumors derived from epithelia and have examined expression level of MMP-26 in normal and abnormal tissues (8,9,15,16,33-35). MMP-26 may contribute to the local invasion of cancer cells since it is located at the leading edge of invading cells (21). It may initiate the tumor invasion and carcinogenesis.

Since cell models that overexpress MMP-26 have not been well established to date, we established an MMP-26 overexpression model and have analyzed the function of MMP-26 *in vitro* and *in vivo* to elucidate its potential function

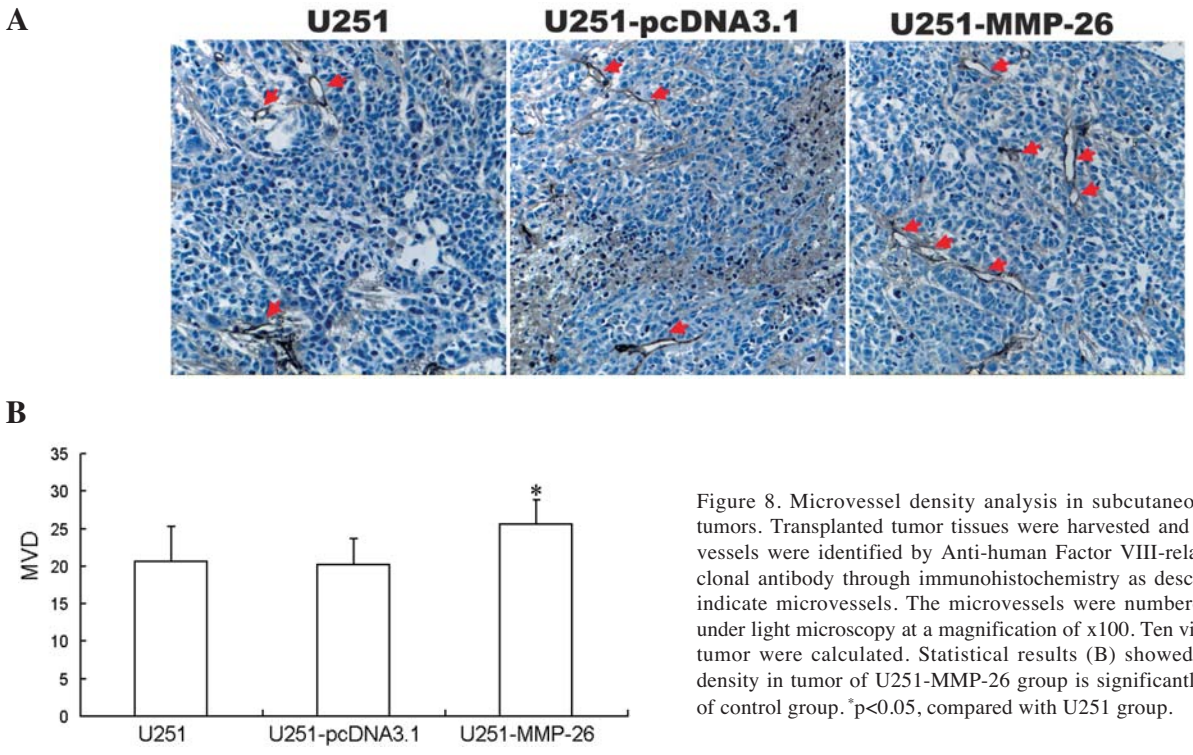



Figure 8. Microvessel density analysis in subcutaneously transplanted tumors. Transplanted tumor tissues were harvested and sectioned. Microvessels were identified by Anti-human Factor VIII-related antigen polyclonal antibody through immunohistochemistry as described. The arrows indicate microvessels. The microvessels were numbered and quantified under light microscopy at a magnification of x100. Ten visual fields of each tumor were calculated. Statistical results (B) showed that microvessel density in tumor of U251-MMP-26 group is significantly higher than that of control group. *p<0.05, compared with U251 group.

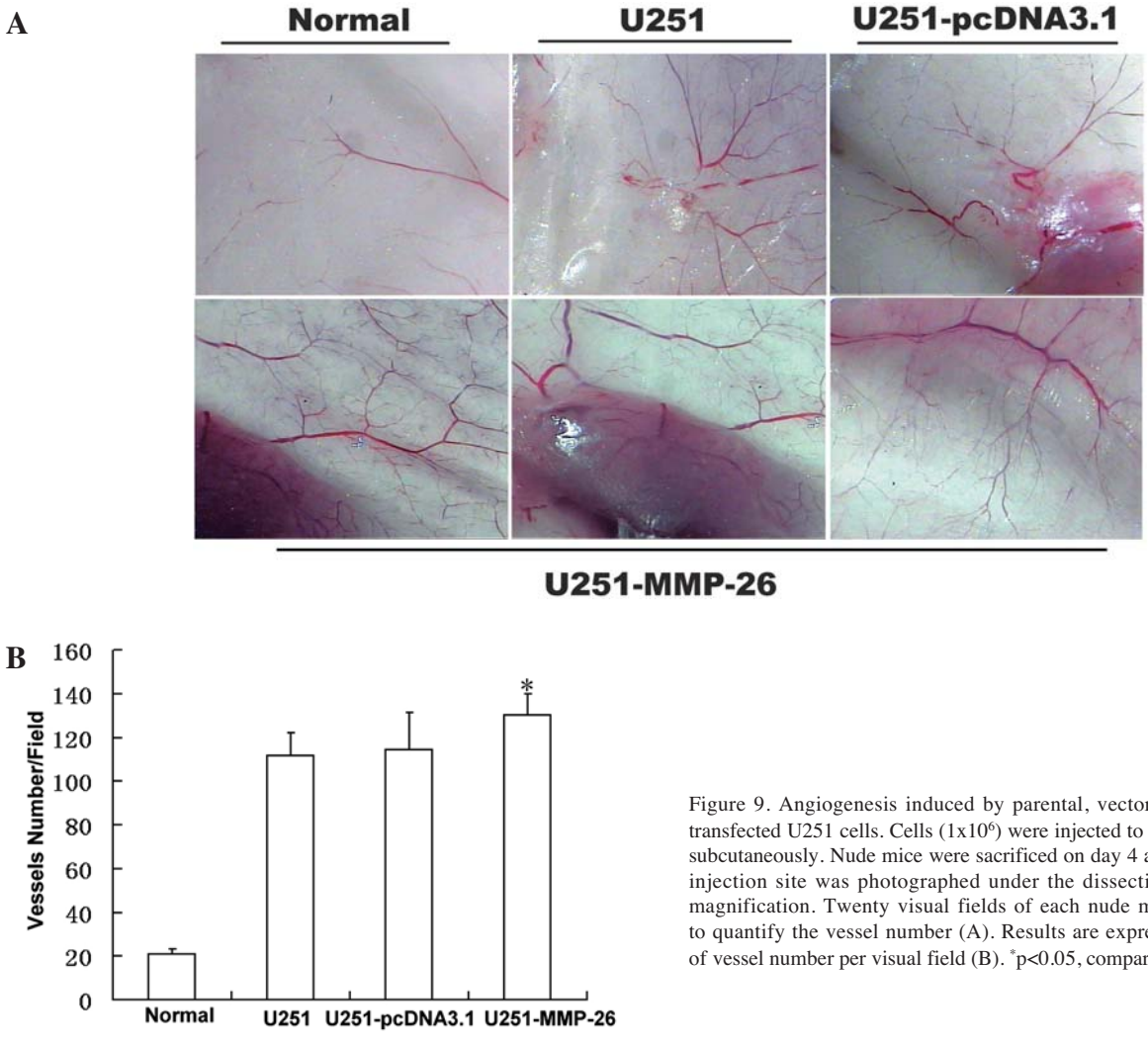



Figure 9. Angiogenesis induced by parental, vector and MMP-26 stable transfected U251 cells. Cells ($1x10^6$) were injected to the back of nude mice subcutaneously. Nude mice were sacrificed on day 4 after the injection. The injection site was photographed under the dissecting microscope at x7 magnification. Twenty visual fields of each nude mouse were employed to quantify the vessel number (A). Results are expressed as mean (± SD) of vessel number per visual field (B). *p<0.05, compared with U251 group.

in tumor progression. We first sought evidence for MMP-26 expression in tumor cell lines of epithelial and non-epithelial origin. On the basis that the non-epithelial human glioma cell line U251 was found to lack expression of MMP-26, it was chosen to be our target transfection cell line.

After MMP-26 had been successfully overexpressed in U251 cells, we focused our study on its contribution to the ability of cells to invade through *in vitro* assays of adherence, spreading and migration. The adherence and spreading assay showed that the MMP-26 transfected cells attached to extracellar matrix more quickly in the Matrigel and Fibronectin coated wells. The percentage of adherent cells that showed features of spreading increased in MMP-26 transfected cells. The migration assay showed that the MMP-26 transfected cells migrated faster than cells in the control group. The Boyden chamber assay showed that MMP-26 expression significantly enhanced the ability of U251 cells to invade through Matrigel.

Published research has shown that MMPs can promote tumor invasion through tissue barriers by degrading extracellular matrix and disrupting the basement membrane (36). MMP-26 can degrade most of the ECM components including fibronectin, vitronectin, fibrinogen, type IV collagen, gelatin, and non-ECM proteins including α1-proteinase inhibitor and growth factor-binding protein 1 *in vitro* (11-13). Our study showed that MMP-26 expression contributes to the adherence and spreading ability of U251 cells on extracellular matrix components which contributes to the invasion process *in vitro*. Other laboratories have also used gene transfection to upregulate MMP-26 in esophageal squamous cell carcinoma TE-1 cells (21) and downregulate it in human prostate cancer ARCaP cells (14). Invasion ability through ECM components was enhanced in MMP-26 overexpressing TE-1 cells and suppressed by its downregulation in ARCaP cells. These results agree with our findings.

In order to further elucidate the effects of MMP-26 on tumor invasion *in vivo*, we injected the transfected and non-transfected U251 cells subcutaneously into nude mice. We demonstrated that MMP-26 can promote the local invasion of U251 cells into blood vessels, fibrous connective tissue, and muscle tissue around the tumor. Indeed, MMP-26 expressing tumor cells broke through the peritoneum and infiltrated organs including liver, spleen and pancreas in the peritoneal cavity. Our findings argue that MMP-26 can promote invasion of U251 cells *in vitro* and *in vivo*. The mechanism needs to be further studied. One possibility is that MMP-26 can degrade extracellular matrix and release biologically-active factors which can regulate tumor cell features (11,12). Another possibility is that MMP-26 can function by activating proMMP-9 through cleavage of Ala93-Met94 site of the proenzyme, which is a powerful stimulator of tumor invasion and metastasis (14,21). TIMP-4 is thought to be the endogenous inhibitor of MMP-26, the expression balance and location between MMP-26 and TIMP-4 also may contribute to the invasive ability of tumor cells (19,20,22,34,37).

Angiogenesis is a key process in tumor development and progression (38). MMPs play important roles in angiogenesis positively and/or negatively. MMPs can release extracellular matrix-bound angiogenic growth factors, regulate growth factors and receptors, expose proangiogenic binding sites in the extracellular matrix, generate proangiogenic extracellular matrix component fragments, and cleave endothelial cell-cell adhesions (3,5). MMPs also generate endogenous anti-angiogenic factors to suppress angiogenesis (5). To our knowledge, there has been no research on the effects of MMP-26 in tumor angiogenesis. However, the unique expression pattern of MMP-26 in cycling human endometrium (16,39) and tumors hints that it may have effects on angiogenesis. In our study, we detected the effects of MMP-26 on angiogenesis. We employed microvessel density assays of transplanted tumors in nude mice and assays of tumor cell induced angiogenesis in nude mice. We found that the microvessel density in MMP-26 transfected U251 cell transplanted tumors was significantly higher than that in control groups. The vessels induced by MMP-26 transfected U251 cells were abundant in number, wider in diameter, and longer in total length than vessels induced by control cells. They also formed a vascular network. All of these features demonstrate that MMP-26 can induce tumor angiogenesis efficiently. The mechanism of this needs further elucidation. However, this result suggests a functional role of MMP-26 in tumor development and also raises the possibility that MMP-26 may contribute to normal physiological processes (15,33,34).

In summary, we established an MMP-26 overexpressing cell line and demonstrated that MMP-26 contributed to the invasion process and angiogenesis of U251 cells *in vitro* and *in vivo*. To our knowledge, no prior research has examined the effects of MMP-26 on tumor progression *in vivo* or angiogenesis. Our results suggest MMP-26 has important roles in tumor progression. However, the regulation of MMP-26 is still unclear. To elucidate this mechanism is our next aim.

## Acknowledgements

This study was supported by The National Natural Science Foundation of China (Grants: NSFC No. 30470662 and NSFC No. 30870970). Jilin Provincial Science and Technology Projects: The Prominent Youth Research project (20050118) and the Bethune Medicine Project (200705358). We also acknowledge Dr F. William Orr for his important suggestions on manuscript organization.

## References

1. Strongin AY: Mislocalization and unconventional functions of cellular MMPs in cancer. Cancer Metastasis Rev 25: 87-98, 2006.
2. Malemud CJ: Matrix metalloproteinases (MMPs) in health and disease: an overview. Front Biosci 11: 1696-1701, 2006.
3. Van Hinsbergh VW and Koolwijk P: Endothelial sprouting and angiogenesis: matrix metalloproteinases in the lead. Cardiovasc Res 78: 203-212, 2008.
4. Heissig B, Hattori K, Friedrich M, Rafii S and Werb Z: Angiogenesis: vascular remodeling of the extracellular matrix involves metalloproteinases. Curr Opin Hematol 10: 136-141, 2003.
5. Rundhaug JE: Matrix metalloproteinases and angiogenesis. J Cell Mol Med 9: 267-285, 2005.
6. De Coignac AB, Elson G, Delneste Y, Magistrelli G, Jeannin P, Aubry JP, Berthier O, Schmitt D, Bonnefoy JY anf Gauchat JF: Cloning of MMP-26. A novel matrilysin-like proteinase. Eur J Biochem 267: 3323-3329, 2000.
7. Uria JA and Lopez-Otin C: Matrilysin-2, a new matrix metalloproteinase expressed in human tumors and showing the minimal domain organization required for secretion, latency, and activity. Cancer Res 60: 4745-4751, 2000.

8. Park HI, Ni J, Gerkema FE, Liu D, Belozerov VE and Sang QX: Identification and characterization of human endometase (Matrix metalloproteinase-26) from endometrial tumor. J Biol Chem 275: 20540-20544, 2000.
9. Marchenko GN, Ratnikov BI, Rozanov DV, Godzik A, Deryugina EI and Strongin AY: Characterization of matrix metalloproteinase-26, a novel metalloproteinase widely expressed in cancer cells of epithelial origin. Biochem J 356: 705-718, 2001.
10. Marchenko ND, Marchenko GN and Strongin AY: Unconventional activation mechanisms of MMP-26, a human matrix metalloproteinase with a unique PHCGXXD cysteine-switch motif. J Biol Chem 277: 18967-18972, 2002.
11. Park HI, Turk BE, Gerkema FE, Cantley LC and Sang QX: Peptide substrate specificities and protein cleavage sites of human endometase/matrilysin-2/matrix metalloproteinase-26. J Biol Chem 277: 35168-35175, 2002.
12. Li W, Savinov AY, Rozanov DV, Golubkov VS, Hedayat H, Postnova TI, Golubkova NV, Linli Y, Krajewski S and Strongin AY: Matrix metalloproteinase-26 is associated with estrogen-dependent malignancies and targets alpha1-antitrypsin serpin. Cancer Res 64: 8657-8665, 2004.
13. Park HI, Jin Y, Hurst DR, Monroe CA, Lee S, Schwartz MA and Sang QX: The intermediate S1' pocket of the endometase/matrilysin-2 active site revealed by enzyme inhibition kinetic studies, protein sequence analyses, and homology modeling. J Biol Chem 278: 51646-51653, 2003.
14. Zhao YG, Xiao AZ, Newcomer RG, Park HI, Kang T, Chung LW, Swanson MG, Zhau HE, Kurhanewicz J and Sang QX: Activation of pro-gelatinase B by endometase/matrilysin-2 promotes invasion of human prostate cancer cells. J Biol Chem 278: 15056-15064, 2003.
15. Li Q, Wang H, Zhao Y, Lin H, Sang QA and Zhu C: Identification and specific expression of matrix metalloproteinase-26 in rhesus monkey endometrium during early pregnancy. Mol Hum Reprod 8: 934-940, 2002.
16. Goffin F, Munaut C, Frankenne F, Perrier D'Hauterive S, Beliard A, Fridman V, Nervo P, Colige A and Foidart JM: Expression pattern of metalloproteinases and tissue inhibitors of matrix-metalloproteinases in cycling human endometrium. Biol Reprod 69: 976-984, 2003.
17. Isaka K, Nishi H, Nakai H, Nakada T, Feng Li Y, Ebihara Y and Takayama M: Matrix metalloproteinase-26 is expressed in human endometrium but not in endometrial carcinoma. Cancer 97: 79-89, 2003.
18. Ahokas K, Skoog T, Suomela S, Jeskanen L, Impola U, Isaka K and Saarialho-Kere U: Matrilysin-2 (matrix metalloproteinase-26) is upregulated in keratinocytes during wound repair and early skin carcinogenesis. J Invest Dermatol 124: 849-856, 2005.
19. Tunuguntla R, Ripley D, Sang QX and Chegini N: Expression of matrix metalloproteinase-26 and tissue inhibitors of metalloproteinases TIMP-3 and -4 in benign endometrium and endometrial cancer. Gynecol Oncol 89: 453-459, 2003.
20. Lee S, Desai KK, Iczkowski KA, Newcomer RG, Wu KJ, Zhao YG, Tan WW, Roycik MD and Sang QX: Coordinated peak expression of MMP-26 and TIMP-4 in preinvasive human prostate tumor. Cell Res 16: 750-758, 2006.
21. Yamamoto H, Vinitketkumnuen A, Adachi Y, Taniguchi H, Hirata T, Miyamoto N, Nosho K, Imsumran A, Fujita M, Hosokawa M, Hinoda Y and Imai K: Association of matrilysin-2 (MMP-26) expression with tumor progression and activation of MMP-9 in esophageal squamous cell carcinoma. Carcinogenesis 25: 2353-2360, 2004.
22. Zhao YG, Xiao AZ, Park HI, Newcomer RG, Yan M, Man YG, Heffelfinger SC and Sang QX: Endometase/matrilysin-2 in human breast ductal carcinoma in situ and its inhibition by tissue inhibitors of metalloproteinases-2 and -4: a putative role in the initiation of breast cancer invasion. Cancer Res 64: 590-598, 2004.
23. Ahokas K, Karjalainen-Lindsberg ML, Sihvo E, Isaka K, Salo J and Saarialho-Kere U: Matrix metalloproteinases 21 and 26 are differentially expressed in esophageal squamous cell cancer. Tumour Biol 27: 133-141, 2006.
24. Li D: Peaking of MMP-26 and TIMP-4 marks invasive transition in prostate cancer. Cell Res 16: 741, 2006.
25. Savinov AY, Remacle AG, Golubkov VS, Krajewska M, Kennedy S, Duffy MJ, Rozanov DV, Krajewski S and Strongin AY: Matrix metalloproteinase 26 proteolysis of the NH2-terminal domain of the estrogen receptor beta correlates with the survival of breast cancer patients. Cancer Res 66: 2716-2724, 2006.
26. Lee S, Park HI and Sang QX: Calcium regulates tertiary structure and enzymatic activity of human endometase/matrilysin-2 and its role in promoting human breast cancer cell invasion. Biochem J 403: 31-42, 2007.
27. Li GH, Wei H, Chen ZT, Lv SQ, Yin CL and Wang DL: STAT3 silencing with lentivirus inhibits growth and induces apoptosis and differentiation of U251 cells. J Neurooncol (In press).
28. Aesoy R, Sanchez BC, Norum JH, Lewensohn R, Viktorsson K and Linderholm B: An autocrine VEGF/VEGFR2 and p38 signaling loop confers resistance to 4-hydroxytamoxifen in MCF-7 breast cancer cells. Mol Cancer Res 6: 1630-1638, 2008.
29. Smith S, Sepkovic D, Bradlow HL and Auborn KJ: 3,3'-Diindolylmethane and genistein decrease the adverse effects of estrogen in LNCaP and PC-3 prostate cancer cells. J Nutr 138: 2379-2385, 2008.
30. Mycielska ME, Broke-Smith TP, Palmer CP, Beckerman R, Nastos T, Erguler K and Djamgoz MB: Citrate enhances in vitro metastatic behaviours of PC-3M human prostate cancer cells: status of endogenous citrate and dependence on aconitase and fatty acid synthase. Int J Biochem Cell Biol 38: 1766-1777, 2006.
31. Chittezhath M, Deep G, Singh RP, Agarwal C and Agarwal R: Silibinin inhibits cytokine-induced signaling cascades and down-regulates inducible nitric oxide synthase in human lung carcinoma A549 cells. Mol Cancer Ther 7: 1817-1826, 2008.
32. Ripley D, Tunuguntla R, Susi L and Chegini N: Expression of matrix metalloproteinase-26 and tissue inhibitors of metalloproteinase-3 and -4 in normal ovary and ovarian carcinoma. Int J Gynecol Cancer 16: 1794-1800, 2006.
33. Fortunato SJ and Menon R: Screening of novel matrix metalloproteinases (MMPs) in human fetal membranes. J Assist Reprod Genet 19: 483-486, 2002.
34. Zhang J, Cao YJ, Zhao YG, Sang QX and Duan EK: Expression of matrix metalloproteinase-26 and tissue inhibitor of metalloproteinase-4 in human normal cytotrophoblast cells and a choriocarcinoma cell line, JEG-3. Mol Hum Reprod 8: 659-666, 2002.
35. Bar-Or A, Nuttall RK, Duddy M, Alter A, Kim HJ, Ifergan I, Pennington CJ, Bourgoin P, Edwards DR and Yong VW: Analyses of all matrix metalloproteinase members in leukocytes emphasize monocytes as major inflammatory mediators in multiple sclerosis. Brain 126: 2738-2749, 2003.
36. Curran S and Murray GI: Matrix metalloproteinases: molecular aspects of their roles in tumour invasion and metastasis. Eur J Cancer 36: 1621-1630, 2000.
37. Pilka R, Domanski H, Hansson S, Eriksson P and Casslen B: Endometrial TIMP-4 mRNA is high at midcycle and in hyperplasia, but down-regulated in malignant tumours. Coordinated expression with MMP-26. Mol Hum Reprod 10: 641-650, 2004.
38. Kerbel RS: Tumor angiogenesis. N Engl J Med 358: 2039-2049, 2008.
39. Pilka R, Whatling C, Domanski H, Hansson S, Eriksson P and Casslen B: Epithelial expression of matrix metalloproteinase-26 is elevated at mid-cycle in the human endometrium. Mol Hum Reprod 9: 271-277, 2003.