

Overexpression of ACE2 produces antitumor effects via inhibition of angiogenesis and tumor cell invasion *in vivo* and *in vitro*

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Abstract. Angiotensin II (AngII) is a multifunctional bioactive peptide in the renin-angiotensin system (RAS). Angiotensin-converting enzyme 2 (ACE2) is a newly identified component of RAS. The role of AngII and ACE2 in the metastasis of non-small cell lung cancer (NSCLC) and the effects on matrix metalloproteinases (MMPs) are still unknown. In the present study, the anti-invasive effect and mechanism of ACE2 were investigated *in vitro* and *in vivo*. Results of a transwell assay showed that the overexpression of ACE2 reduces the invasive ability of A549 cells *in vitro*. According to the results of qRT-PCR and Western blot analysis, the inhibitory role of ACE2 was mediated through the down-regulation of MMP-2 and MMP-9. Additionally, we confirmed that the overexpression of ACE2 inhibited cell growth and VEGFa production while simultaneously suppressing ACE and angiotensin II type 1 receptor (AT1R) expression in human lung cancer xenografts. These results suggest that the overexpression of ACE2 may potentially suppress the invasion and angiogenesis of NSCLC.

Introduction

Lung cancer is the most common human neoplasm in both developed and developing countries (1,2). Approximately 40% of lung cancers are adenocarcinomas, which are the

predominant form in both the US and Asia (3). Most patients with lung adenocarcinoma have advanced disease due to the highly metastatic properties of this cancer, making such patients non-surgical candidates. Thus, the metastatic nature of lung adenocarcinoma has been a major barrier to successful treatment and improved patient outcomes.

The renin-angiotensin system (RAS) is important in regulating cardiovascular homeostasis and blood pressure (4). Angiotensin II (AngII), a biologically active octapeptide in RAS, and its biologic effect are mediated via binding to two subtypes of receptors. The receptor subtypes include type 1 (AT1R) and type 2 (AT2R), both of which belong to the G-protein-coupled receptor superfamily (5). Angiotensin-converting enzyme 2 (ACE2) is a newly identified component of RAS, with 42% amino acid homology to ACE. ACE2 converts AngII to Ang-(1-7) (6), a peptide with vasodilator and anti-proliferative properties.

Tumor invasion and migration require a complex cascade of events involving a finely tuned interplay between malignant cells and multiple host factors. Excess extracellular matrix (ECM) degradation is one of the hallmarks of tumor invasion and migration (7). Matrix metalloproteinases (MMPs) are a large family of at least 20 zinc-dependent neutral endopeptidases that together can degrade all known components of the ECM. Among the human MMPs, MMP-2 (also named gelatinase-A) and MMP-9 (gelatinase-B) show substrate-specificity toward type IV collagen, the major component of the basement membrane. Expression of these two MMPs is strongly linked to tumor metastasis in various types of human cancer (8).

Emerging data suggest that, in addition to systemically produced angiotensin, the tumor environment contains all RAS components necessary to produce angiotensin locally. Importantly, the local RAS system contributes significantly to tumor angiogenesis and progression (9-11). Studies have shown that ACEI reduces non-small cell lung cancer (NSCLC) growth and angiogenesis (12,13) and that lung metastases are reduced by ACEI and angiotensin II type 1 receptor blocker (ARB), via inhibition of MMP-2 expression in mice bearing Lewis lung carcinoma (14). Gallagher and Tallant (15) demonstrated that Ang-(1-7) inhibit lung tumor growth *in vivo* and *in vitro* as

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well as reduce vascular endothelial growth factor expression in human lung cancer xenografts, thereby inhibiting tumor angiogenesis (16). Various *in vivo* studies strongly suggest that a major role of ACE2 involves the generation of Ang-(1-7) from AngII (17-19). We previously reported that overexpression of ACE2 exhibits a protective effect by inhibiting cell growth and VEGFa production in A549 cells *in vitro* (20). However, the role of AngII and ACE2 in the migration and invasion of NSCLC and the effects on MMPs are still unknown.

In the present study, AngII stimulated the expression of MMP-2 and MMP-9 in A549 human adenocarcinoma cells. In addition, we hypothesized that the ability of ACE2 to reduce the invasive nature of NSCLC is mediated by the down-regulation of MMP-2 and MMP-9. We confirmed that the overexpression of ACE2 inhibited cell growth and VEGFa production while simultaneously suppressing the expression of other RAS components in human lung cancer xenografts.

Materials and methods

Retroviral vector construct and transduction. The pcDNA3.1 vector containing human ACE2 cDNA was kindly provided by Dr Paul McCray (University of Iowa, Iowa City, IA). They were ligated into the *Bgl*II/*Xho*I site of the pMSCV plasmid, which expresses human ACE2. 293T cells were transfected with DNA (4 μ g pMD-gag-pol, 4 μ g pMD-VSVG and 4 μ g retroviral vector pMSCV-ACE2 or pMSCV) using the Lipofectamine 2000 reagent (Invitrogen Corp., Carlsbad, CA). The replication retrovirus was collected after 48 h and used to infect A549 cells using polybrene (Sigma Corp., Cream Ridge, NJ) and centrifugation was carried out. Infected cells were selected after receiving 4 μ g/ml puromycin for 2 weeks. The resistant clones were isolated by limit dilution and dispatched to new dishes. The obtained cells were subsequently named vector and MSCV-ACE2 cells, respectively.

Tumor growth assays. Six- to eight-week-old BALB/c athymic nude mice (Chinese Academy of Sciences, Shanghai, China) were used. Animal experiments were performed in accordance with the institutional guidelines of the University Committee on the Use and Care of Animals. Each mice was inoculated in the flank with 1×10^6 A549 cells infected with MSCV (n=6) or A549 cells infected with MSCV-ACE2 (n=6). Tumor growth was monitored at 3-day intervals by measuring the tumor diameters using a vernier caliper. Tumor volume was determined based on the following formula: Tumor volume = [(major axis) \times (minor axis)²] \times 1/2. After a 31-day follow-up period, mice were euthanized, and the tumors were removed. Harvested tumors were cut into 2 pieces, one of which was placed in liquid nitrogen and frozen at -80°C and the other was fixed using 4% paraformaldehyde and then embedded in paraffin.

Immunohistochemistry. Paraffin-embedded tissue sections (5 μ m) were deparaffinized, microwaved for 5 min for antigen retrieval, and then incubated with a monoclonal mouse anti-human antibody against Ki-67 (1:50, clone MIB-1; Dako), goat anti-human antibody against VEGFa (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), MMP-2 (1:200, AF902, R&D) and MMP-9 (1:200, AF911; R&D) overnight at 4°C.

They were rinsed with TBS and incubated in biotinylated IgG antibody for 90 min at room temperature (RT). After incubation in streptavidin-HRP (1:300) for 30 min at RT, the sections were developed with 3,3'-diaminobenzidine (DAB) substrate and counterstained with hematoxylin. The scoring of NSCLC samples was performed by a histopathologist, by counting at least 500 tumor cells in 5 different visual fields for each specimen. The intensity of the immunoreactivity (intensity score) was stratified and scored as follows: 1, $\leq 25\%$ of cells exhibited positive staining; 2, 26-50% of cells exhibited positive staining; 3, 51-75% of cells exhibited positive staining; and 4, $> 75\%$ of cells exhibited positive staining. An intensity score of ≥ 2 with at least 50% of tumor cells staining positive was indicative of patients with high expression; intensity score of < 2 with $< 50\%$ of tumor cells staining positive was indicative of patients with low expression. The total score for each cellular compartment was obtained by calculating the product of intensity and the percentage of staining.

Quantitation of microvessels. For the measurement of microvessel density (MVD), tumor sections were first deparaffinized, endogenous peroxide production was blocked with 1% hydrogen peroxidase inhibitor and non-specific protein binding was blocked with 10% rabbit serum and 2% bovine serum for 30 min. Sections were then incubated overnight at 4°C with the anti-mouse CD31 antibody (Dako, Glostrup, Denmark). Finally, sections were incubated for 30 min with peroxidase-labeled biotinylated anti-rat antibody at RT. After incubation in streptavidin-HRP (1:300) for 30 min at RT, sections were developed with DAB substrate and counterstained with hematoxylin. Briefly, the immunostained sections were initially screened at low magnifications (x40 and x100) to identify hot spots, which were the areas of highest neovascularization. Any yellow-brown stained endothelial cell or endothelial cell cluster that was clearly separate from adjacent microvessels, tumor cells, and other connective tissue elements was considered a single, countable microvessel. Within the hot spot area, the stained microvessels were counted in a single high-power (x200) field, and the average vessel count in 3 hot spots was considered the MVD value. All counts were performed by three investigators in a blinded manner.

Cell culture and detection of AngII concentration. A549 lung cancer cells (Cell Bank of Chinese Academic of Science, Shanghai) were maintained in Ham's F12 medium with 10% FBS, 100 mg/ml penicillin and 100 U/ml streptomycin using media and growth reagents from Gibco (Gibco-BRL, Grand Island, NY). Cells were prepared at 37°C in 5% CO₂. To measure the influence of different concentrations of AngII or overexpression of ACE2 on MMP production, A549 cells infected with MSCV or MSCV-ACE2 were plated in a 12-well plate. After a 24-h incubation in 10% FBS, the cells were serum starved for 24 h, and incubated in serum-free medium for 24 h. AngII levels were measured in the supernatants of the A549 cells. After a 24-h incubation with 10% FBS, A549 cells overexpressing ACE2 and control cells were serum starved for 24 h. Cells were then incubated in serum-free medium for 24 h. The culture media were collected, and AngII levels were measured using radioimmunoassay, carried out at the Ruijin Hypertension Institute Laboratory.

In vitro cell invasion assay. Cell invasion was evaluated using 24-well Matrigel invasion chambers (Becton-Dickinson, Franklin Lakes, NJ). Cells were suspended in the upper chamber at a density of 4×10^4 cells in 200 μ l medium with various concentrations of AngII. The lower chamber contained 750 μ l Ham's F12 supplemented with 10% FCS as a chemo-attractant. After incubation for 16 h, the remaining tumor cells on the upper surface of the filters were removed by wiping with cotton swabs, and invading cells on the lower surface were stained using May-Grünwald Giemsa. The cells on the lower surface of the filters were counted using a microscope. Data were obtained from three individual experiments in triplicate.

Real-time quantitative reverse transcription analysis. MMP-2 and MMP-9 mRNA expression was examined by real-time quantitative reverse transcription-based polymerase chain reaction (qRT-PCR) in untreated A549 cells and those stimulated with AngII (10^{-7} and 10^{-6} mol/l) for 15 min *in vitro*. AT1R, ACE, MMP-2 and MMP-9 mRNA expression was also examined in the MSCV-ACE2 and vector groups, in the A549 nude mouse models. RNA was extracted from cells with TRIzol reagent and treated with DNase (Promega, Madison, WI). Complementary DNA was synthesized using a cDNA synthesis kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Fluorescence qRT-PCR was performed with the double-stranded DNA dye SYBR Green (PCR Core Reagents; PE Biosystems, Warrington, UK), using the ABI PRISM 7300 system (Applied Biosystems). The SYBR Green assay contained 1 μ l 10X SYBR Green PCR buffer, 0.8 μ l deoxynucleotide triphosphate (dNTP) mixture, 0.1 μ l AmpErase UNG (1 U/ μ l), 0.05 μ l AmpliTaq Gold DNA polymerase (5 U/ μ l), 1.2 μ l $MgCl_2$ (25 mM), 0.1 μ l forward and reverse primer (20 μ M), 1 μ l cDNA and 5.65 μ l double distilled H_2O . PCR was initiated with one cycle at 50°C for 2 min and 95°C for 10 min proceeded by 45 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 3 sec. All data were analyzed using ABI PRISM SDS 2.0 software (Perkin Elmer, Wellesley, MA). Using the ΔC_t method, GAPDH was co-amplified to normalize the amount of RNA added to the reaction, and the data were subjected to cycling threshold analysis. PCR was repeated at least three times. The primers used in this study were as follows: ACE forward 5'-CCGATCTGGCAGAACTTC-3' and reverse 5'-GTGTTCCAGATCGTCCTC-3'; AT1-R forward 5'-ATA TTCCCCAAAAGCCAAATCC-3' and reverse 5'-TCAACC TCAAAACATGGTGCAG-3'; MMP-2 forward 5'-CATTTG GCGGACTGTGAC-3' and reverse 5'-GGGTGCTGGCTG AGTAGAT-3'; MMP-9 forward 5'-TGCCAGTTTCCATTC ATCTTCC-3' and reverse 5'-CCCATCACCGTCGAGTC AGC-3'; GAPDH forward 5'-ATTGCCGACAGGATGC AGA-3' and reverse 5'-GAGTACTTGCGCTCAGGAGGA-3'.

Western blot analysis. MMP-2 and MMP-9 protein levels in A549 cells were studied after treatment with or without AngII (10^{-7} and 10^{-6} mol/l) for 24 h. Expression of MMP-2 and MMP-9 was also assessed in the A549 cells overexpressing ACE2 and compared to the control cells. After being treated with a protein assay reagent (Bio-Rad Laboratories, Hercules, CA), the soluble protein was separated on 10% SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore, Billerica,

MA). The MMP-2, MMP-9 and β -actin monoclonal (1:10,000; Sigma Corp., Cream Ridge, NJ) antibodies were used to detect MMP-2, MMP-9 and β -actin protein, respectively. The immunoreactive bands were visualized with ECL plus reagent (Amersham Biosciences, Piscataway, NJ).

Analysis of MMP-2 and MMP-9 activity using gelatin zymography. Conditioned medium from an equal number of cells was prepared as described above and separated by 10% acrylamide gels containing 0.1% gelatin (Invitrogen). The gels were incubated in 2.5% Triton X-100 solution at RT with gentle agitation to remove SDS and were subsequently soaked in reaction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, and 0.5 mM $ZnCl_2$) at 37°C overnight. After incubation, the gels were stained for 1 h with staining solution (0.1% Coomassie Brilliant Blue, 30% methanol and 10% acetic acid) and destained in the same solution, but without Coomassie Brilliant Blue. Gelatinolytic activity of MMP-2 and MMP-9 was visualized as a clear band against a dark background of stained gelatin.

Statistical analysis. Results are expressed as the means \pm SD of a number of experiments as indicated. The difference between experimental and control values was assessed by the Independent samples test and ANOVA. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

AngII enhances invasion and MMP production in A549 lung cancer cells. As shown in Fig. 1A, 10^{-7} and 10^{-6} mol/l of AngII significantly increased the invasive ability of the treated A549 cells. Analysis of qRT-PCR data of MMP-2 and MMP-9 mRNA, corrected for GAPDH as an internal control, showed that 10^{-7} and 10^{-6} mol/l of AngII significantly increased expression of MMP-2 and MMP-9 mRNA (Fig. 1B and C; $P < 0.05$). In the Western blot analysis, the expression of MMP-2 and MMP-9 protein in the A549 cells treated with 10^{-7} and 10^{-6} mol/l was significantly higher than the levels in the control cells (Fig. 1D and E). The gelatin zymography assay demonstrated that active MMP-2 (72 kDa) and MMP-9 (92 kDa) expression was increased in the culture medium of the A549 cells treated with 10^{-6} mol/l (Fig. 1F and G). AngII caused a modest increase in MMP-2 mRNA and significantly increased MMP-9 mRNA. However, Western blot analysis and gelatin zymography assay showed that MMP-9 protein and the activity level were increased in the AngII-treated A549 cells more significantly than MMP-2.

Overexpression of ACE2 inhibits invasion and MMP production in vitro. We investigated whether the overexpression of ACE2 inhibits tumor invasion and MMP production in A549 lung cancer cells. Infection of A549 cells with MSCV-ACE2 resulted in robust ACE2 expression as determined by Western blot analysis at 72 h. No significant ACE2 protein expression was observed in the A549 cells infected with the vector in a similar manner or in the normal lung tissue used as a positive control (Fig. 2A). The AngII concentration in the supernatants of the A549 cells decreased in the MSCV-ACE2 group as compared to the vector cells (Fig. 2B). The *in vitro* transwell

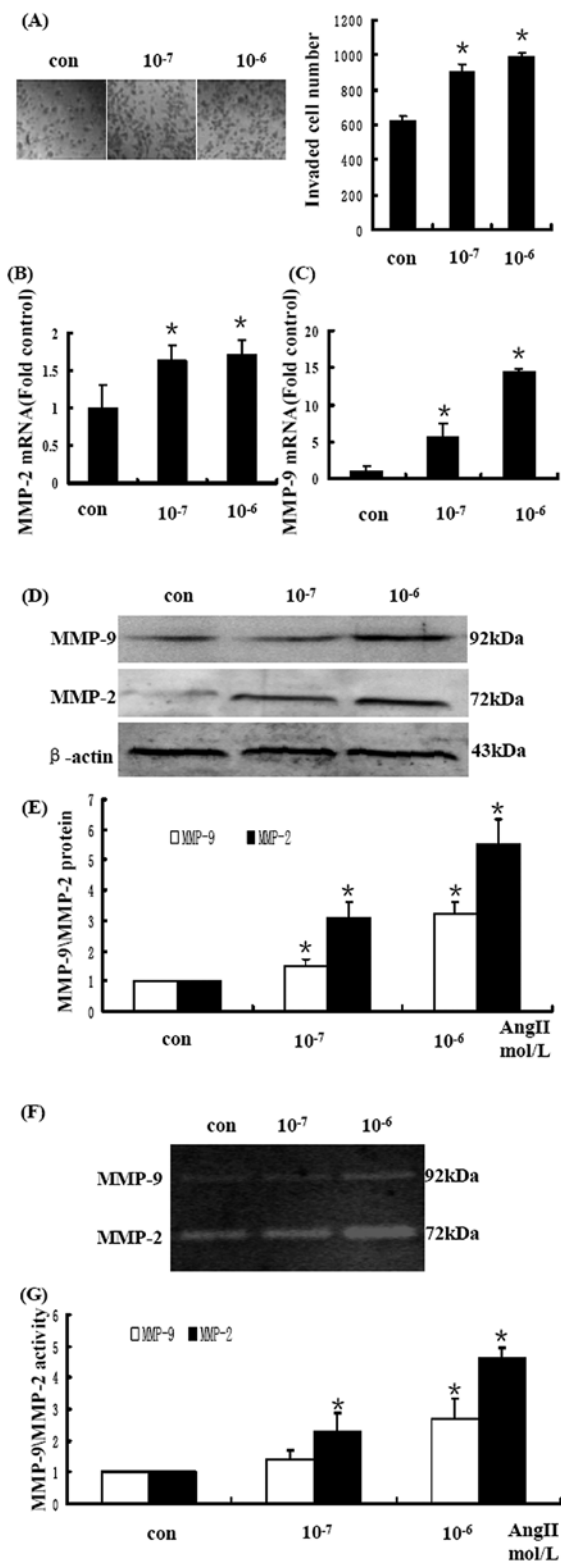


Figure 1. Ang II enhances the invasion and MMP production in A549 lung cancer cells. (A) An *in vitro* invasion assay was performed using 24-well transwell units coated with Matrigel. The number of invading cells was determined 24 h after cell seeding. Effects of different concentrations of AngII on production of MMP-2 (B) and MMP-9 (C) mRNA were assessed (means \pm SD, $n=3$) * $p<0.05$. (D) Expression of MMP-2 and MMP-9 protein in cultured A549 cells treated with different concentrations of AngII is shown. (E) Densitometric ratios of MMP-2 and MMP-9 proteins to β -actin. (F) Zymography assay was performed, and the gelatinolytic activity of active MMP-2 (72 kDa) and MMP-9 (92 kDa) was visualized as clear bands against a dark background of stained gelatin. Values in (E) and (G) (means \pm SD) are fold increases. Value of cells without AngII (control) treatment was taken as 1. All experiments were repeated in triplicate with similar results. * $p<0.05$ vs. control.

assays indicated that the ACE2-overexpressing cells exhibited lower invasive ability than the vector cells (Fig. 2C). Western blot analysis showed that the MMP-2 and MMP-9 protein levels decreased in the A549 cells infected with MSCV-ACE2 compared with expression levels in the vector cells (Fig. 2D and E). Gelatin zymography assays also demonstrated that active MMP-2 (72 kDa) and MMP-9 (92 kDa) levels were decreased in the A549 cells infected with MSCV-ACE2 compared with expression in the cells infected with the vector (Fig. 2F and G).

Overexpression of ACE2 inhibits growth of A549 tumor cells in vivo. To investigate whether the overexpression of ACE2 inhibits pre-established tumor growth in nude mice, all animals were observed and tumors were measured during a 31-day follow-up period. Analysis of Western blot data showed that expression of ACE2 protein in the MSCV-ACE2 group was significantly higher than in the vector group (Fig. 3A). Tumor size in the MSCV-ACE2 group was significantly smaller than that in the vector group (Fig. 3B). The total weight of disseminated tumors in the MSCV-ACE2 group was significantly reduced compared with the vector group (Fig. 3C). Tumor growth curves indicated that the growth in the MSCV-ACE2 group was significantly reduced than that in the vector group (Fig. 3D). These data are in agreement with our previous observations and demonstrated that overexpression of ACE2 significantly inhibited tumor growth in the A549 nude mouse model. In addition, the proliferation-related gene Ki-67 was examined by immunohistochemistry, and the results showed that Ki-67 staining intensity was weaker in the MSCV-ACE2 group than that in the vector group (Fig. 3E).

Overexpression of ACE2 reduces tumor-associated angiogenesis in vivo. The overexpression of ACE2 inhibited VEGFa protein production *in vivo*, according to immunohistochemical data from A549 tumor tissue xenografts (Fig. 4A). We hypothesized that ACE2 influences neovascularization in tumors. Therefore, we investigated microvessel density (MVD) in tumor sections and found that CD31 (endothelial specific marker)-positive staining was lower in tumors of the MSCV-ACE2 group than in the vector group (Fig. 4B), and MVD was significantly higher in the vector group compared with the MSCV-ACE2 group (Fig. 4C).

Effects of ACE2 overexpression on RAS components and MMP production in vivo. Analysis of qRT-PCR data of ACE, AT1R, MMP-2, and MMP-9 mRNA, corrected for GAPDH as an internal control, showed that the overexpression of ACE2 significantly decreased expression of ACE (Fig. 5A), AT1R (Fig. 5B), MMP-2 (Fig. 5C) and MMP-9 (Fig. 5D) mRNA in the MSCV-ACE2 group. Immunohistochemical analysis showed that the expression of MMP-2 and MMP-9 proteins in the MSCV-ACE2 group was significantly lower than that in the vector group (Fig. 5E and F).

Discussion

To our knowledge, this study is the first to show that overexpression of ACE2 inhibits tumor invasion, metastasis and MMP production. Several critical findings should be emphasized including the enhancement of invasion of A549 cells by

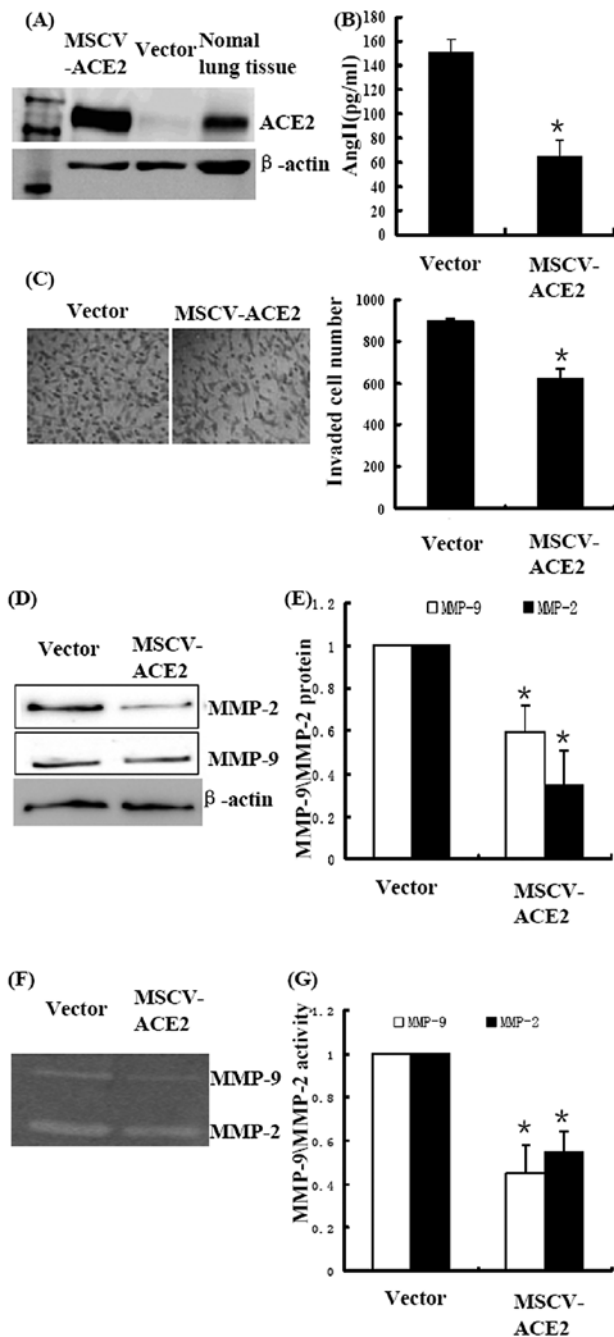


Figure 2. Overexpression of ACE2 inhibits invasion and MMP production *in vitro*. (A) ACE2 expression increased in the MSCV-ACE2 group compared to the vector group as determined by Western blot analysis. (B) AngII concentration in the supernatants of A549 cells decreased in the MSCV-ACE2 group compared to the vector group. (C) The MSCV-ACE2 group showed significantly decreased invasive ability compared to the vector group *in vitro*. (D) MMP-2 and MMP-9 protein production was decreased in A549 cells infected with MSCV-ACE2 compared with the vector group as determined by Western blot analysis. (E) Densitometric ratios of MMP-2 and MMP-9 proteins to β -actin. (F) Zymography assay was performed, and the gelatinolytic activity showed that active MMP-2 (72 kDa) and MMP-9 (92 kDa) were decreased in the MSCV-ACE2 group compared to the vector group *in vitro*. Values in (E) and (G) (means \pm SD) are fold decreases; value for cells of the vector group was taken as 1. All experiments were repeated in triplicate with similar results. * $p < 0.05$ vs. vector.

AngII *in vitro* and the involvement of MMP-2 and MMP-9. Additionally, we demonstrated that ACE2 suppresses cancer cell invasion and migration, potentially by decreasing MMP-2

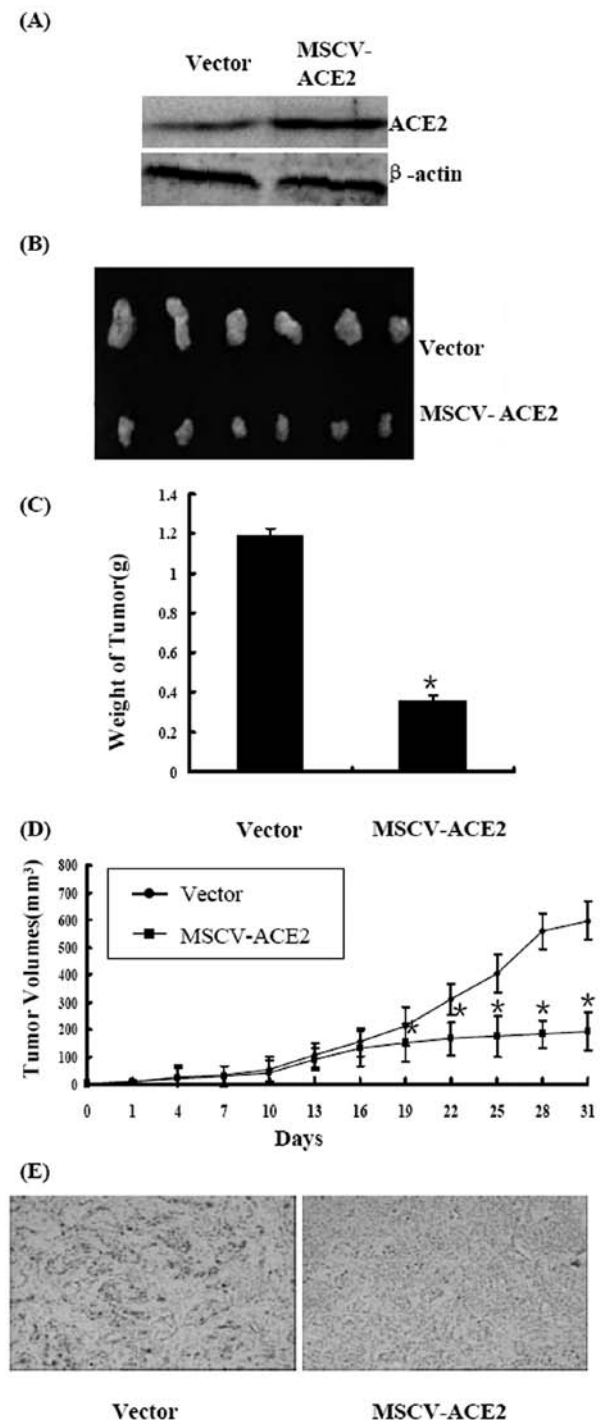


Figure 3. Overexpression of ACE2 inhibits growth of A549 tumor cells *in vivo*. Nude mice were xenografted s.c. with human lung A549 cells infected with MSCV or A549 cells infected with MSCV-ACE2 (10^6 cells per animal) for 31 days. Tumors were removed for qRT-PCR and Western blot analysis (A), and images of the tumor samples from both groups are shown (B). The impact of the overexpression of ACE2 on tumor weight (C) and volume (D) is shown. (E) Ki-67 staining intensity was weaker in the MSCV-ACE2 group than in the vector group by immunohistochemistry analysis. Data points represent the means \pm SD of six mice per group. Statistically significant differences are indicated in (C) for tumor weight and (D) for tumor volume (* $p < 0.05$ vs. control).

and MMP-9 activity. These data confirm our previous *in vivo* study in which we revealed that the overexpression of ACE2 inhibits the growth of A549 tumor cells and reduces tumor-

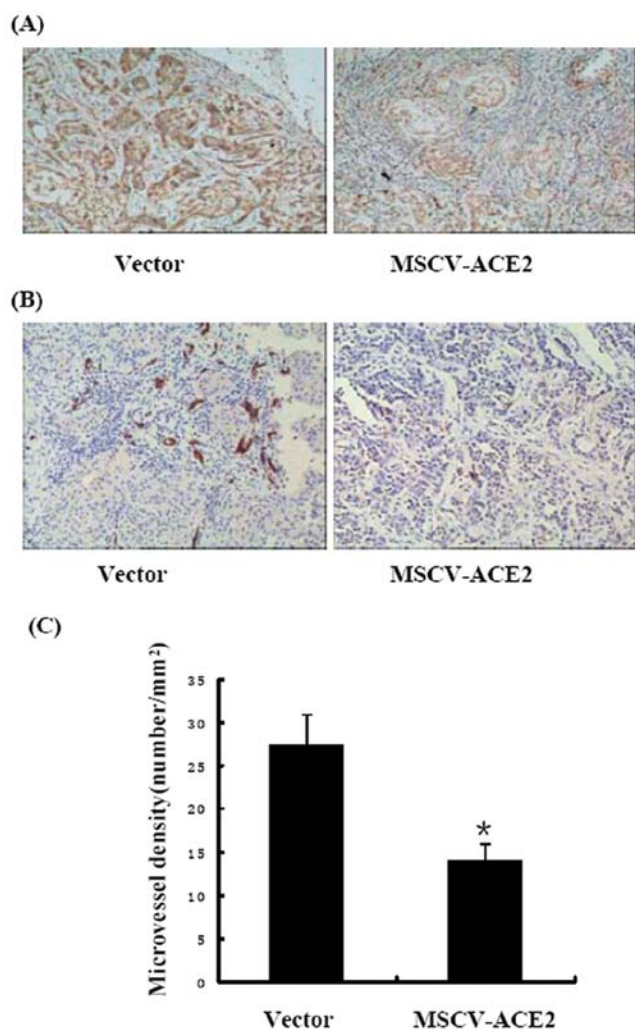


Figure 4. Overexpression of ACE2 reduces tumor-associated angiogenesis *in vivo*. (A) VEGFa staining intensity was weaker in the MSCV-ACE2 group than in the vector group by immunohistochemistry analysis. (B) Immunohistochemical detection of vascular endothelial cells for CD31 expression. Representative images for the different treatment groups are shown. The positive expression of CD31 is mainly represented as brownish-yellow or brownish granules in vascular endothelial cells. (C) Tumor microvessel density (MVD) in the different treatment groups. The MVD in the MSCV-ACE2 group was significantly lower than that in the vector group. * $p<0.05$.

associated angiogenesis *in vivo*. These novel findings further support the potential of ACE2 as a new strategy for anti-cancer therapy.

The RAS system increases expression of MMPs and modulates the balance between MMPs and tissue inhibitors of MMPs (TIMPs) in many heart and renal diseases (21-24). Previous cancer studies have demonstrated that AngII augments the migration and invasion of choriocarcinoma cells through the AT1R and induces MMP-2 and MMP-9 expression in gastric cancer cells (25-27). Consistent with these effects of AngII, we showed that treatment with AngII significantly increases the invasive ability of A549 lung cancer cells. qRT-PCR, Western blot analysis, and gelatin zymography analyses showed that AngII induces MMP-2 and MMP-9 expression in A549 cells *in vitro*.

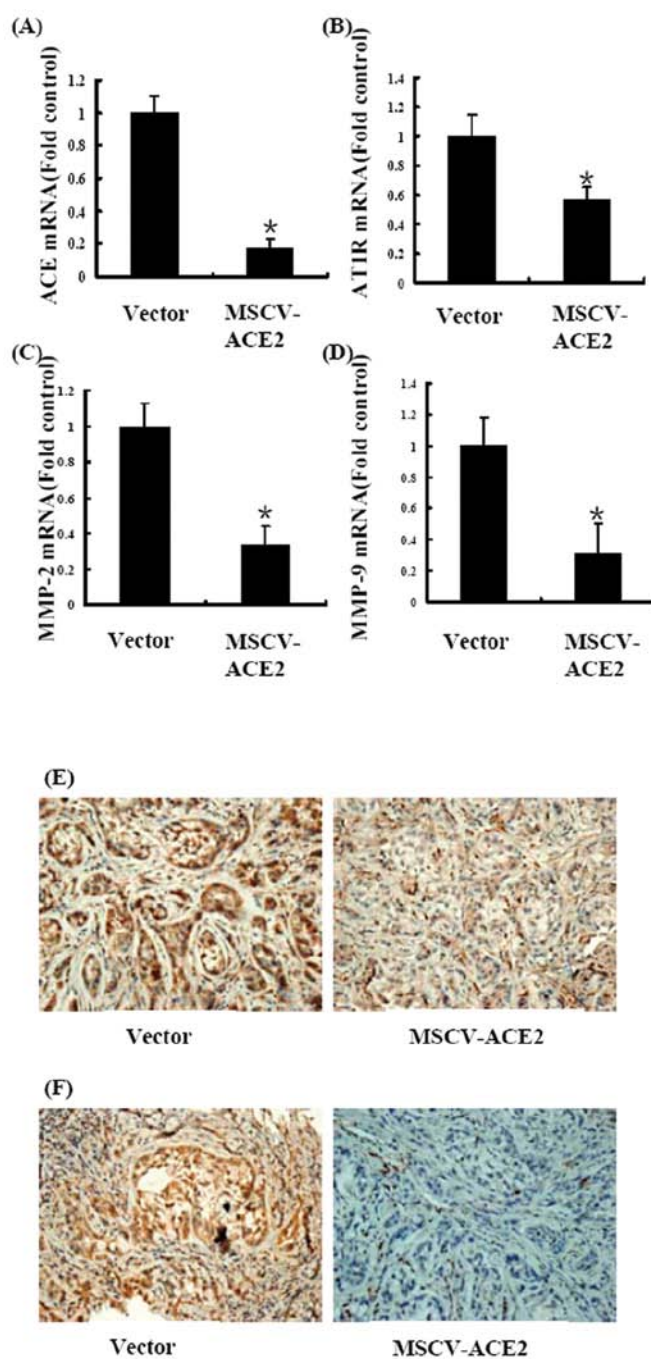


Figure 5. Effects of ACE2 overexpression on RAS components and MMP productions *in vivo*. Effects of ACE2 overexpression on production of ACE (A), AT1R (B), MMP-2 (C) and MMP-9 (D) mRNA in an A549 nude mouse model; (means \pm SD, $n=3$) * $p<0.05$. Expression of MMP-2 (E) and MMP-9 (F); staining intensity was weaker in the MSCV-ACE2 group than in the vector group as determined by immunohistochemical analysis.

Previous studies have demonstrated moderate ACE2 expression in the lungs of both humans (28) and mice (29), with high levels of ACE2 in the kidney, heart, testis, and small intestine of both species (29-31). In the human lung, immunostaining found that ACE2 was localized to endothelial and smooth muscle cells of large and small blood vessels, as well as type I and II alveolar epithelial cells and bronchial epithelial cells (32,33). In addition to AngI

and AngII, ACE2 was found to hydrolyze apelin-13, neurotensin-(1-11), dynorphin A-(1-13), β -casomorphin-(1-7), and ghrelin. Although bradykinin is not hydrolyzed by ACE2 similar to ACE, its metabolite des-Arg (9)-(bradykinin), can be hydrolyzed by ACE2. These peptides have a negative effect on constriction of the myocardium. It was initially hypothesized that disruption of the delicate balance between ACE and ACE2 may result in abnormal blood pressure control. ACE2 may protect against increases in blood pressure and, conversely, ACE2 deficiency could lead to hypertension.

Our previous studies showed that the overexpression of ACE2 inhibits cell growth and VEGFa production in A549 cells (20). In the present study, our data demonstrated that the overexpression of ACE2 inhibits tumor growth and tumor-associated angiogenesis of A549 cells *in vivo*. We performed immunohistochemical analysis of proliferating cells (Ki-67) in tumor samples from the MSCV-ACE2 group and vector group. Quantification of the Ki-67-positive cells showed a decreased number of dividing tumor cells in the MSCV-ACE2 group compared with the vector group. Our immunohistochemical analysis demonstrated that VEGFa protein and microvessel density were lower in tumors in the MSCV-ACE2 group than that of the vector group, which is notable when one considers that MVD is reported to be a useful prognostic factor of cancer (34).

We assessed whether the overexpression of ACE2 inhibits A549 cell invasion, MMP production and/or change in the expression of RAS components *in vitro* and *in vivo*. Many studies have shown that angiotensin-converting enzyme inhibitors (ACE-I) and angiotensin II type 1 receptor blockers (ARB) reduce the invasiveness of gastric cancer cells and human gastric cancer xenografts (35,36). These results correspond with evidence that captopril inhibits human glioma cell migration and invasion via a reduction in MMP activity (37). Consistent with our previous *in vitro* data, we found that the overexpression of ACE2 decreased ACE and AT1R mRNA in the MSCV-ACE2 group of the A549 nude mouse model in this study. We also found that the overexpression of ACE2 suppressed A549 cell invasion and MMP-2 and MMP-9 activity *in vitro* and *in vivo*. Our present study also demonstrated that Ang-(1-7) suppress metastasis and MMP production in A549 cells *in vitro* (data not shown). Together, these findings reveal that ACE2 inhibits invasiveness and MMP production by AngII in A549 cells through decreased ACE and AT1R production. Previous studies indicate that the transcription of MMPs is regulated by upstream regulatory sequences, including NF- κ B, AP-1 and Ets-1 binding sites (38,39). The PI3K/Akt signaling pathway also plays a crucial role in MMP-2 and MMP-9 gene regulation, cell survival and tumor cell metastasis (40). However, we did not assess the influence of ACE2 on upstream regulatory sequences of MMPs. Further studies are required to evaluate the upstream signaling pathways of MMPs.

Notably, the ratio of ACE/ACE2 is important. Recent research has shown that an imbalance in this ratio leads to various diseases. Many studies have shown that ACE2 appears to be a negative regulator of the RAS and counterbalances the function of ACE. ACE2 converts AngII to Ang-(1-7), which has the opposite function of AngII. We previously

found that AngII significantly increased expression of AT1R and decreased expression of ACE2. Overexpression of ACE2 decreases AT1R production *in vitro*. In the present research we confirmed that overexpression of ACE2 decreased AT1R production *in vivo* and found that ACE2 overexpression inhibited the expression of ACE. This effect is through the overexpression of ACE2 which decreases AngII production and increases Ang-(1-7) accumulation.

In conclusion, these results suggest the therapeutic potential of ACE2 for controlling tumor progression and metastasis based on its inhibitory effect on the growth and invasion of the A549 adenocarcinoma cancer cell line. However, tumor blood vessels may be constricted and tortuous, thus the effect of ACE2 should be further studied.

Acknowledgements

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