

Piceatannol inhibits migration and invasion of prostate cancer cells: possible mediation by decreased interleukin-6 signaling[☆]

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Abstract

Piceatannol (*trans*-3,4,3',5'-tetrahydroxystilbene) is a polyphenol detected in grapes, red wine and *Rheum undulatum*; it has also been demonstrated to exert anticarcinogenic effects. In this study, in order to determine whether piceatannol inhibits the lung metastasis of prostate cancer cells, MAT-Ly-Lu (MLL) rat prostate cancer cells expressing luciferase were injected into the tail veins of male nude mice. The oral administration of piceatannol (20 mg/kg) significantly inhibited the accumulation of MLL cells in the lungs of these mice. In the cell culture studies, piceatannol was demonstrated to inhibit the basal and epidermal growth factor (EGF)-induced migration and invasion of DU145 cells, in addition to the migration of MLL, PC3 and TRAMP-C2 prostate cancer cells. In DU145 cells, piceatannol attenuated the secretion and messenger RNA levels of matrix metalloproteinase-9, urokinase-type plasminogen activator (uPA) and vascular endothelial growth factor (VEGF). Piceatannol increased the protein levels of tissue inhibitor of metalloproteinase-2 in a concentration-dependent fashion. Additionally, piceatannol inhibited the phosphorylation of signal transducer and activator of transcription (STAT) 3. Furthermore, piceatannol effected reductions in both basal and EGF-induced interleukin (IL)-6 secretion. An IL-6 neutralizing antibody inhibited EGF-induced STAT3 phosphorylation and EGF-stimulated migration of DU145 cells. Interleukin-6 treatment was also shown to enhance the secretion of uPA and VEGF, STAT3 phosphorylation and the migration of DU145 cells; these increases were suppressed by piceatannol. These results demonstrate that the inhibition of IL-6/STAT3 signaling may constitute a mechanism by which piceatannol regulates the expression of proteins involved in regulating the migration and invasion of DU145 cells.

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Keywords: Matrix metalloproteinase; Piceatannol; STAT3; Prostate cancer cells; Interleukin-6

1. Introduction

Most cancer patients die as a result of metastasis, as opposed to the initial tumor. Despite the profound advances that have been made in the clinical treatment of cancer over the past several decades, the

currently available treatment options rarely cure metastatic cancers. Thus, it is necessary to focus greater efforts on the prevention and/or control of metastasis, as opposed to the treatment of metastatic cancer [1]. Prostate cancer is currently the most prevalent non-cutaneous cancer in males in the United States and is also the second leading cause of death from cancer in males [2]. Prostate cancer has also become a more frequently diagnosed disease in Asian countries; this may be attributable to Westernized dietary habits. Prostate cancer patients are treated initially via an androgen deprivation method. However, in the majority of cases, prostate cancer ultimately progresses to a hormone-refractory state, during which androgen deprivation treatment is no longer effective [3]. Thus, the chemoprevention of prostate cancer through dietary agents appears a worthwhile pursuit, as prostate cancer is generally diagnosed in elderly populations.

Piceatannol (*trans*-3,4,3',5'-tetrahydroxystilbene) (Fig. 1) is a naturally occurring polyphenol present in rhubarb, berries, peanuts, sugar cane, wine and the skins of grapes. Piceatannol has

Abbreviations: EGF, epidermal growth factor; IL-6, interleukin-6; MMP, matrix metalloproteinase; STAT, signal transducer and activator of transcription; TIMP, tissue inhibitor of metalloproteinase; uPA, urokinase-type plasminogen activator; VEGF, vascular endothelial growth factor.

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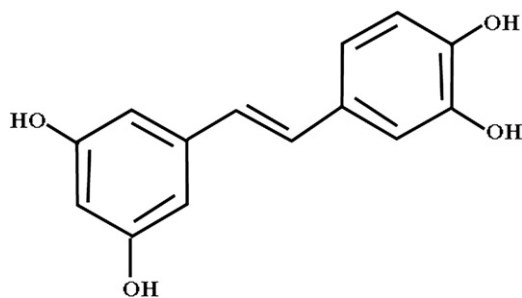


Fig. 1. Structure of piceatannol.

been demonstrated to exert anti-inflammatory, anticarcinogenic and cardioprotective effects (reviewed in Ref. [4]). Additionally, piceatannol has been identified as an inhibitor of the interferon- α -mediated signal transducer and activator of transcription (STAT) 3 [5] as well as the purified thymocyte protein-tyrosine kinase p40 [6].

STATs are the transcription factors that mediate cellular responses to growth factors, peptides and cytokines [7], and STAT3 controls several oncogenic processes, including cell proliferation, cell survival, angiogenesis and immune responses and has emerged as a promising molecular target for cancer therapy (reviewed in Ref. [8]). The suppression of the STAT3 signaling pathway can inhibit angiogenesis via the down-regulation of vascular endothelial growth factor (VEGF) production by tumors and via the inhibition of VEGF and basic fibroblast growth factor-induced signaling in endothelial cells (as reviewed in Ref. [11]). The cytokine interleukin (IL)-6 can activate STAT3 by binding to cell surface receptor tyrosine kinases that are coupled with activated Janus kinases (JAKs). The JAKs then phosphorylate the tyrosine 705 residue in the transactivation domain of the STAT3 protein (reviewed in Ref. [9]). The phosphorylation of tyrosine 705 induces STAT3 dimerization, nuclear translocation, and subsequent binding to STAT3 response elements. The phosphorylation of serine 727 in the transactivation domain contributes to maximal transcriptional activity (reviewed in Ref. [12]). The IL-6-mediated activation of STAT3 has been reported to perform a crucial function in tumor growth, metastasis and angiogenesis [10,11]. The constitutive activation of STAT3 has been previously implicated in the development and progression of a variety of cancers, including prostate cancer [9,11,12].

Piceatannol has been shown to inhibit the metastasis of Lewis lung carcinoma in carcinectomized mice [13]. We, along with other investigators, previously noted that piceatannol induces apoptosis in the human prostate cancer lines DU145 [14] and NRP-154 [15]. We also noted that piceatannol induces G1 cell cycle arrest in DU145 cells [16]. To the best of our knowledge, the effects of piceatannol on prostate cancer metastasis and the mechanisms underlying these effects have yet to be determined in detail. In this study, we demonstrated that piceatannol potently inhibits the metastatic potential of androgen-insensitive DU145 cells. We have also observed that the oral administration of piceatannol at a dose of 20 mg/kg body weight significantly inhibits the metastasis of MAT-Ly-Lu (MLL) prostate cancer cells to the lungs of nude mice upon the injection of MLL cells into the tail veins of the mice.

2. Materials and methods

2.1. Materials

The following reagents were purchased from the indicated suppliers: piceatannol (Sigma, St. Louis, MO, USA); STAT3 antibody (Cell Signaling, Beverly, MA, USA); antibodies against matrix metalloproteinase (MMP)-9, tissue inhibitor of metallopro-

teinase (TIMP)-2 and VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-urokinase-type plasminogen activator (uPA) (Calbiochem, Darmstadt, Germany); VEGF enzyme-linked immunosorbent assay (ELISA) kit, IL-6, IL-6 neutralizing antibody, IL-6 ELISA kit and epidermal growth factor (EGF) (R&D Systems, Minneapolis, MN, USA); antibodies against STAT3 (PY705) and STAT3 (PS727) (BD Biosciences, San Jose, CA, USA). Unless noted otherwise, all other materials were acquired from Sigma.

2.2. Cell culture

The DU145, MAT-Ly-Lu (MLL), PC3 and TRAMP-C2 cell lines were acquired from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM/F12 containing 100 ml/L of fetal bovine serum (FBS) with 100,000 U/L of penicillin and 100 mg/L of streptomycin (Lonza, Walkersville, MD, USA). In order to determine the effects of piceatannol on the numbers of viable cells, we plated cells in multiwell plates with DMEM/F12 containing 100 ml/L of FBS. Prior to piceatannol treatment, the cell monolayers were subjected to 24 h of serum deprivation in DMEM/F12 supplemented with 1% charcoal-stripped FBS. Following serum deprivation, the monolayers were incubated with fresh serum deprivation medium in the absence or presence of the indicated concentrations of piceatannol. The numbers of viable cells were determined via an MTT assay, as described previously [17]. Piceatannol was dissolved in dimethyl sulfoxide (DMSO), and all of the cells were treated with DMSO to a final concentration of 0.01%.

2.3. Migration and invasion assays

For the transwell migration assay, the DU145, MLL, PC3 and TRAMP-C2 cells were subjected to 24 h of serum deprivation in DMEM/F12 supplemented with 1% charcoal-stripped FBS. They were then plated onto filters in 6.5-mm transwell inserts on 24-well plates at 2.5×10^4 cells/filter and treated with various concentrations of piceatannol. Before plating the cells, the lower side of the transwell filter was precoated with 10 μ g of type IV collagen. The lower chamber of the well was filled with DMEM/F12 containing 1% charcoal-stripped FBS and 0.1% BSA in the absence or presence of 10 μ g/L EGF and/or 20 μ g/L IL-6. The cells were incubated for 4 h (DU145 and MLL), 16 h (PC3) and 1 h (TRAMP-C2), and the migrated cells were stained with hematoxylin and eosin (H&E).

For the invasion assay, the same procedures were performed as described in the migration assay, except that the cells were plated onto matrigel-coated transwell filters (Matrigel invasion chamber, BD Biosciences).

For the wound migration assay, DU145 cells were plated in six-well plates at 8×10^5 cells/well in DMEM/F12 containing 100 ml/L of FBS and serum deprived for 24 h in DMEM/F12 supplemented with 1% charcoal-stripped FBS as described previously [18]. Cells at 90% confluence were incubated for 1 h with 1 mg/L of mitomycin C. After mitomycin C treatment, the injury line was made using a yellow tip, and the cell monolayers were rinsed with PBS. The cells were then incubated for 0, 6, 12 or 24 h with 0 or 10 μ mol/L of piceatannol in the absence or presence of 10 μ g/L of EGF in DMEM/F12 containing 1% charcoal-stripped FBS. Cell migration was microscopically measured at the indicated time points, and the measured widths of the injury lines were plotted as percentages of the width at 0 h.

2.4. Gelatin and fibrin zymography

The cells were serum-starved in DMEM/F12 and treated with or without 10 μ g/L of EGF for 48 h in the absence or presence of piceatannol. Conditioned media were collected and concentrated via centrifugal ultrafiltration using a filter unit with a molecular exclusion of 10,000 (Millipore Corporation, Billerica, MA, USA). Proteins in conditioned media were separated via sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Gelatin (2 g/L) was added to the gel for gelatin zymography, and 1.5 g/L of fibrinogen and 5.6 NIH U/L of thrombin were added to the gel for fibrin zymography. The volumes of media loaded onto the gel were then adjusted for equivalent protein levels. After electrophoresis, the gels were washed twice in 50 mmol/L of Tris-HCl containing 25 ml/L of Triton X-100 for 30 min to completely eliminate SDS. The gels were then rinsed twice in zymogen activation buffer (50 mmol/L Tris-HCl, 0.2 g/L Brij 35, 5 mmol/L CaCl₂ and 0.2 mmol/L NaCl for gelatin zymography and 30 mmol/L Tris, pH 7.4, and 0.2 g/L Na₃ for fibrin zymography) and incubated for 48 h at 37°C in the same buffer. After incubation, the gels were stained for 2 h with 2.5 g/L of Coomassie Brilliant Blue R-250 solution and then destained. The conditioned media of HT1080 human fibrosarcoma cells, which secrete pro MMP-9, MMP-2 and uPA [19–21], were used as positive controls.

2.5. Western blot analyses and ELISA

The total cell lysates (50 μ g protein) and concentrated conditioned media were employed for Western blot analyses, as described previously [22]. Signals were detected via enhanced chemiluminescence using Immobilon Western Chemiluminescent HRP Substrate (Millipore). Densitometric analyses were conducted using the Bio-profile Bio-ID application (Vilber-Lourmat, France). The expression levels were normalized to β -actin, and the control levels were set to 100%.

Table 1
Primer sequences used for real-time PCR amplification

mRNA	Primer sequences	Annealing temperature
MMP-9	Sense: GCTCTCCCTGGAGACCTG Antisense: TTTCGACTTCTCCAGCATC	60
β -Actin	Sense: GAGCCGTGTTCTTCCAT Antisense: GTCCAGTTGGTGACGATG	60
VEGF	Sense: ATGCCAAGTGGTCCCAGGCT Antisense: CAGGGTCTCGATTGGATGGC	54
uPA	Sense: GCCATCTACACAGGAGGCCCG Antisense: GGGTAATCAATGAAGCAGTGTG	54
GADPH	Sense: CATCAGCAATGCCTCCTGCA Antisense: CTGTGGTCATGAGTCTTCC	54

The levels of VEGF and IL-6 in conditioned media were estimated using VEGF and IL-6 ELISA kits (R & D System), respectively, in accordance with the manufacturer's instructions.

2.6. Real-time polymerase chain reaction

Total RNA was isolated with an RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA), and complementary DNA was synthesized using 3 μ g of total RNA with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA), as described previously [23]. Matrix metalloproteinase-9, uPA and VEGF messenger RNA (mRNA) were quantified via real-time polymerase chain reaction (PCR) conducted using a Rotergene 3000 PCR apparatus (Corbett Research, Australia). The sequences for primer sets and annealing temperatures for real-time PCR amplification are provided in Table 1. The analysis of the PCR results and the calculation of the relative concentrations were conducted using the Roter-gene software (version 6), and the control levels (0 μ g/L EGF or 0 μ g/L IL-6+0 μ mol/L piceatannol) were set to 1.

2.7. Establishment of MLL cells stably expressing firefly luciferase (MLL-Luc)

The coding region of the firefly luciferase (Luc) gene from pRevTRE-Luc (Clontech) was cloned into the *Bam*HI and *Hind*III sites in pcDNA3.1/Hygro(+) (Invitrogen). The MLL cells were transfected with the resultant pcDNA3.1/Hygro-Luc using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's protocol.

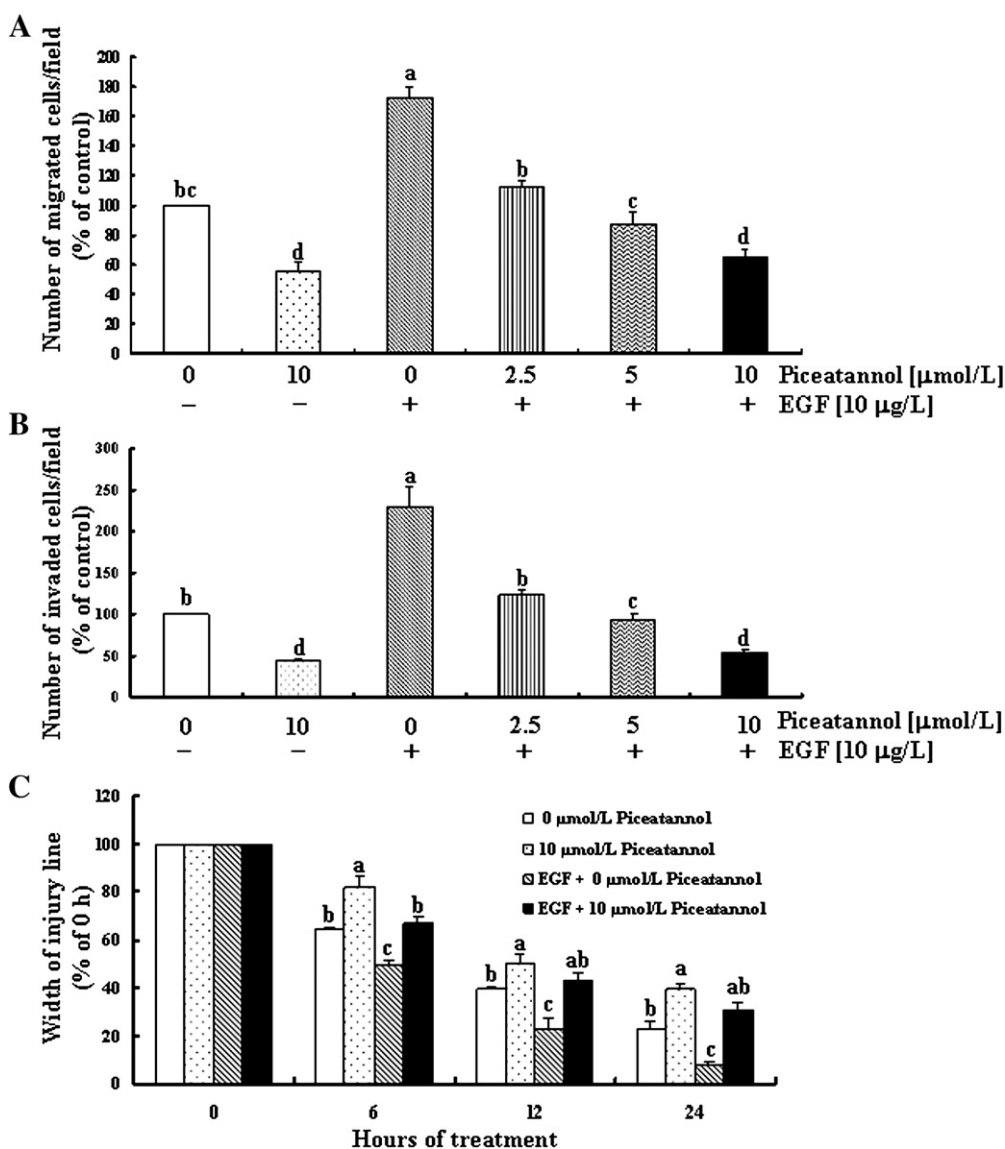


Fig. 2. Piceatannol reduces the migration and invasion of DU145 cells. The migration (A) and invasion (B) of DU145 cells through a type IV collagen-coated (A) and a matrigel-coated (B) transwell filter, respectively, were assessed with or without 10 μ g/L of EGF in the presence of 0–10 μ mol/L of piceatannol. Cells were incubated for 4 h. The migrated (A) and invaded (B) cells were quantified by counting the H&E-stained cells. Each bar represents the mean \pm S.E.M. of three independent experiments. (C) At 90% confluence, DU145 cells were treated with 1 mg/L of mitomycin C, and the injury line was delineated with a yellow tip. The cells were then incubated in DMEM/F12 containing 1% charcoal-stripped FBS with or without 10 μ mol/L of piceatannol in the absence or presence of 10 μ g/L of EGF for 0, 6, 12 or 24 h. The measured widths of the injury lines were plotted as percentages of the width at 0 h. Each bar represents the mean \pm S.E.M. ($n=3$). Means at a time without a common letter differ; $P<0.05$.

The MLL-Luc cells were selected with 200 mg/L of hygromycin B for 3 weeks and then maintained with 20 mg/L of hygromycin B.

2.8. Tumor xenograft bioluminescent imaging

Four-week-old male Balb/c nude mice were purchased from Orient Bio Inc. (Gapyeong, Korea) and were permitted to acclimate under laboratory conditions for 1 week and fed on a nonpurified commercial mouse diet (Superfeed Co., Wonju, Korea) and water ad libitum. For the formation of lung metastasis, 1×10^5 MLL-Luc cells were injected into the lateral tail vein in 0.1 ml of PBS. One day after the injection of MLL-Luc cells, the mice were divided randomly into three groups and subjected to daily oral gavage with vehicle (corn oil), 10 mg/kg/d of piceatannol or 20 mg/kg/d of piceatannol. Lung metastasis involvement was monitored using an *in vivo* imaging system (IVIS-200, Xenogen Corp., Alameda, CA, USA) every 3 days. Prior to *in vivo* imaging, D-luciferin was intraperitoneally injected at 150 mg/kg. After 10 min, mice were anesthetized with isoflurane/oxygen and placed on the imaging stage. Signal intensity was quantified as the sum of all detected photon counts per second within the lung region after subtracting the background luminescence. All procedures involving mice, such as housing and care, and all experimental protocols were approved by the Institutional Animal Care and Use Committee of Hallym University (Hallym 2009-126).

2.9. Statistical analysis

For all studies, three independent experiments were performed with separate batches of cells and animals. The results were expressed as means \pm S.E.M. and then analyzed via analysis of variance. Differences among the treatment groups were evaluated via Duncan's multiple range test. Means were considered significantly different at $P < .05$. All statistical analyses were performed using the SAS System for Windows V 9.1 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Piceatannol inhibits the migration and invasion of prostate cancer cells

As we have previously noted that EGF induces the migration, invasion and adhesion of DU145 cells [18], we cultured cells in the absence or presence of EGF to determine whether piceatannol inhibits the migration and invasion of DU145 cells. The results from the transwell (Fig. 2A) and wound (Fig. 2C) migration assays showed that piceatannol significantly inhibited both the basal and EGF-induced migration of DU145 cells. As the MTT assay showed that the viability of DU145 cells was unaltered at 4 h after the addition of 2.5–10 μ mol/L of piceatannol (data not shown), the transwell filter migration assay was conducted for 4 h to eliminate the effects of piceatannol on cell viability. Additionally, the wound migration assay was conducted in the presence of mitomycin C to eliminate the contribution of cell proliferation. The transwell invasion assay was also conducted using a matrigel-coated filter, demonstrating that piceatannol inhibited the basal and EGF-stimulated invasion of DU145 cells (Fig. 2B). The inhibition of EGF-induced cell migration and invasion was shown to occur in a piceatannol dose-dependent manner. The effect of piceatannol on the migration of other prostate cancer cells was evaluated in the absence or presence of EGF. Piceatannol significantly inhibited the migration of MLL rat prostate cancer cells, PC3 human prostate cancer cells and TRAMP-C2 mouse prostate cancer cells, in both the absence and the presence of EGF (Table 2).

3.2. Piceatannol reduces the secretion of MMP-9 and uPA but increases the secretion of TIMP-2 in DU145 cells

In an effort to determine whether EGF and piceatannol alter MMP secretion in DU145 cells, we collected conditioned media over 48 h and conducted gelatin zymography. On the zymogram, the activities of pro and active MMP-9 were found to have increased in the EGF-treated cells (10 μ g/L EGF+0 μ mol/L piceatannol) relative to the control cells (0 μ g/L EGF+0 μ mol/L piceatannol), and piceatannol reduced the activity of pro and active MMP-9 in the presence of EGF.

Table 2
Effect of piceatannol on migration of various prostate cancer cells

EGF (10 μ g/L)	Piceatannol (μ mol/L)	MLL cells	PC3 cells % of control ¹	TRAMP-C2 cells
–	0	100 \pm 0 ^b	100 \pm 0 ^{cd}	100 \pm 0 ^b
–	10	72 \pm 3 ^d	85 \pm 5 ^d	54 \pm 2 ^d
+	0	172 \pm 7 ^a	162 \pm 9 ^a	132 \pm 5 ^a
+	2.5	132 \pm 5 ^b	125 \pm 5 ^b	89 \pm 7 ^{bc}
+	5	107 \pm 5 ^c	109 \pm 7 ^{bc}	82 \pm 6 ^c
+	10	78 \pm 4 ^d	100 \pm 6 ^{cd}	55 \pm 2 ^d

The migration of prostate cancer cells through a type IV collagen-coated transwell filter was evaluated with or without 10 μ g/L of EGF in the presence of 0–10 μ mol/L of piceatannol. MLL, PC3 and TRAMP-C2 cells were incubated for 4, 16 and 1 h, respectively. The migrated cells were quantified as described in the Materials and Methods section. Values are expressed as the means \pm S.E.M. of three independent experiments. Means without a common letter a,b,c,d in the same column differ significantly; $P < .05$.

In the absence of EGF, piceatannol significantly inhibited the activity of active MMP-9, but the reduction in the activity of pro MMP-9 due to piceatannol treatment was not statistically significant (Fig. 3A). The results of Western blot analysis confirmed that piceatannol reduces the levels of pro-MMP-9 in a dose-dependent fashion in the presence of EGF (Fig. 3B). Results from real-time reverse transcriptase PCR analysis demonstrated that piceatannol reduced the steady-state levels of MMP-9 mRNA only in the presence of EGF (Fig. 3C). On the gelatin zymogram, the activity of 72-kDa pro MMP-2 and 45-kDa MMP-2 activation byproducts [18] was increased by EGF and reduced by piceatannol in both the absence and the presence of EGF. The results of Western blot analysis revealed that TIMP-2 secretion was not affected by EGF, but was increased as a result of piceatannol treatment (Fig. 3B).

The results of fibrin zymography showed that EGF increased the activities of high-molecular-weight and low-molecular-weight uPA, and piceatannol inhibited these activities regardless of EGF treatment (Fig. 4A). The results of Western blot analysis demonstrated that the secretion of pro and active uPA was increased by EGF, and piceatannol reduced uPA secretion in both the absence and the presence of EGF (Fig. 4B). The levels of uPA transcripts were increased by EGF and reduced by piceatannol treatment (Fig. 4C). In order to characterize the role of uPA in the invasion of DU145 cells, we conducted invasion assays in the absence or presence of aprotinin (2.5 or 5 μ mol/L), a serine protease inhibitor, and noted that aprotinin significantly inhibited the invasion of DU145 cells (data not shown). These results indicate that the secretion of uPA, a serine protease, into medium plays a role in the invasion of DU145 cells.

3.3. Piceatannol inhibits VEGF secretion in DU145 cells

We previously noted that EGF increases the secretion of VEGF by DU145 cells [18]. The results of VEGF ELISA (Fig. 5A) and Western blot analysis (Fig. 5B) demonstrated that piceatannol inhibited basal and EGF-induced VEGF secretion by DU145 cells. The levels of VEGF mRNA changed in parallel with the levels of VEGF protein (Fig. 5C).

3.4. Piceatannol inhibits IL-6 secretion in DU145 cells

We attempted to determine whether EGF induces STAT3 activation in DU145 cells. The application of 15 min of EGF treatment to the DU145 cells led to an increase in phosphorylation on the Ser727 residue of STAT3, but had no effects on phosphorylation on Tyr705. Piceatannol inhibited phosphorylation on Ser727 and Tyr705, regardless of whether or not the cells were treated with EGF (Fig. 6A). We assessed the possibility that EGF induces IL-6 secretion,

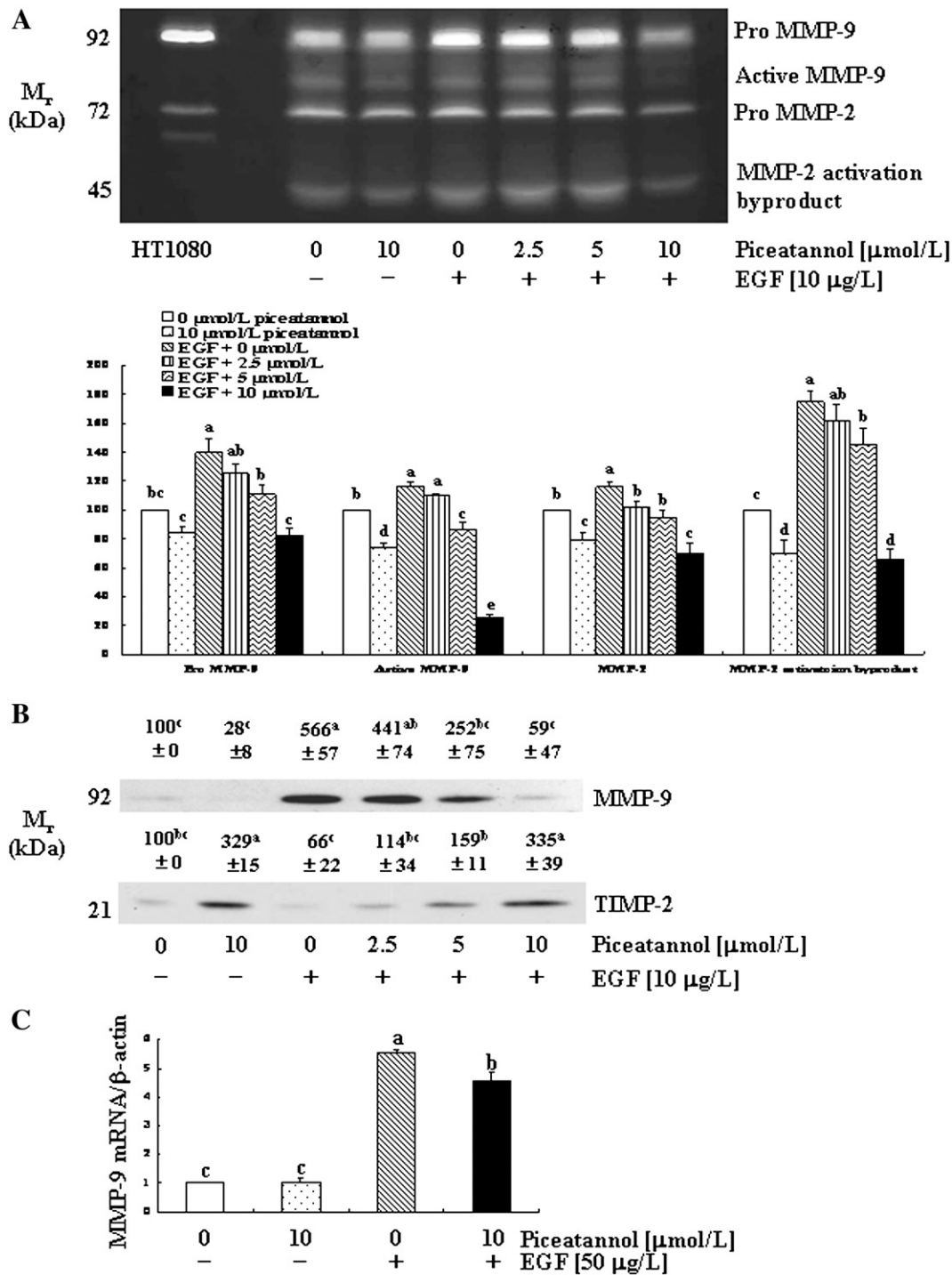


Fig. 3. Piceatannol reduces MMP-9 secretion and increases TIMP-2 secretion in DU145 cells. Serum-starved DU145 cells were incubated with 0–10 $\mu\text{mol/L}$ of piceatannol in DMEM/F12 in the absence or presence of 10 $\mu\text{g/L}$ of EGF for 48 h. Forty-eight-hour conditioned media were collected and concentrated for gelatin zymography (A) and Western blotting (B). The volumes of media loaded onto the gel were adjusted for equivalent protein levels. In the first lane of (A), serum-free HT 1080 cell-conditioned medium was loaded as a positive control. Photographs of the Coomassie-Blue-stained gel (A) and chemiluminescent detection of the blots (B), which were representative of three independent experiments, are shown. The relative abundance of each band was quantified, and the control levels (0 $\mu\text{g/ml}$ EGF+0 $\mu\text{mol/L}$ piceatannol) were set at 100%. (A) Each bar represents the mean \pm S.E.M. ($n=3$). (B) The adjusted mean \pm S.E.M. ($n=3$) of each band is shown above each blot. (C) Serum-starved cells were incubated for 12 h with piceatannol and/or EGF. Total RNA was isolated and reverse transcribed, and real-time PCR was conducted. The expression of MMP-9 mRNA was normalized with that of β -actin. Each bar represents the mean \pm S.E.M. ($n=3$). Means without a common letter differ; $P<.05$.

which in turn induces STAT3 activation; this leads to an increase in cell migration. The results of IL-6 ELISA applied to DU145 cell-conditioned media revealed that the treatment of DU145 cells with EGF for 2 h resulted in a marked increase in IL-6 secretion, and piceatannol inhibited both basal and EGF-induced IL-6 secretion

(Fig. 6B). Additionally, 2 h of EGF treatment applied to the cells resulted in phosphorylation on both Tyr705 and Ser727 of STAT3, and 10 $\mu\text{mol/L}$ of piceatannol inhibited phosphorylation on both residues. Similar to what was observed in the piceatannol-treated cells, EGF-induced phosphorylations on both the Tyr705 and Ser727 of STAT3

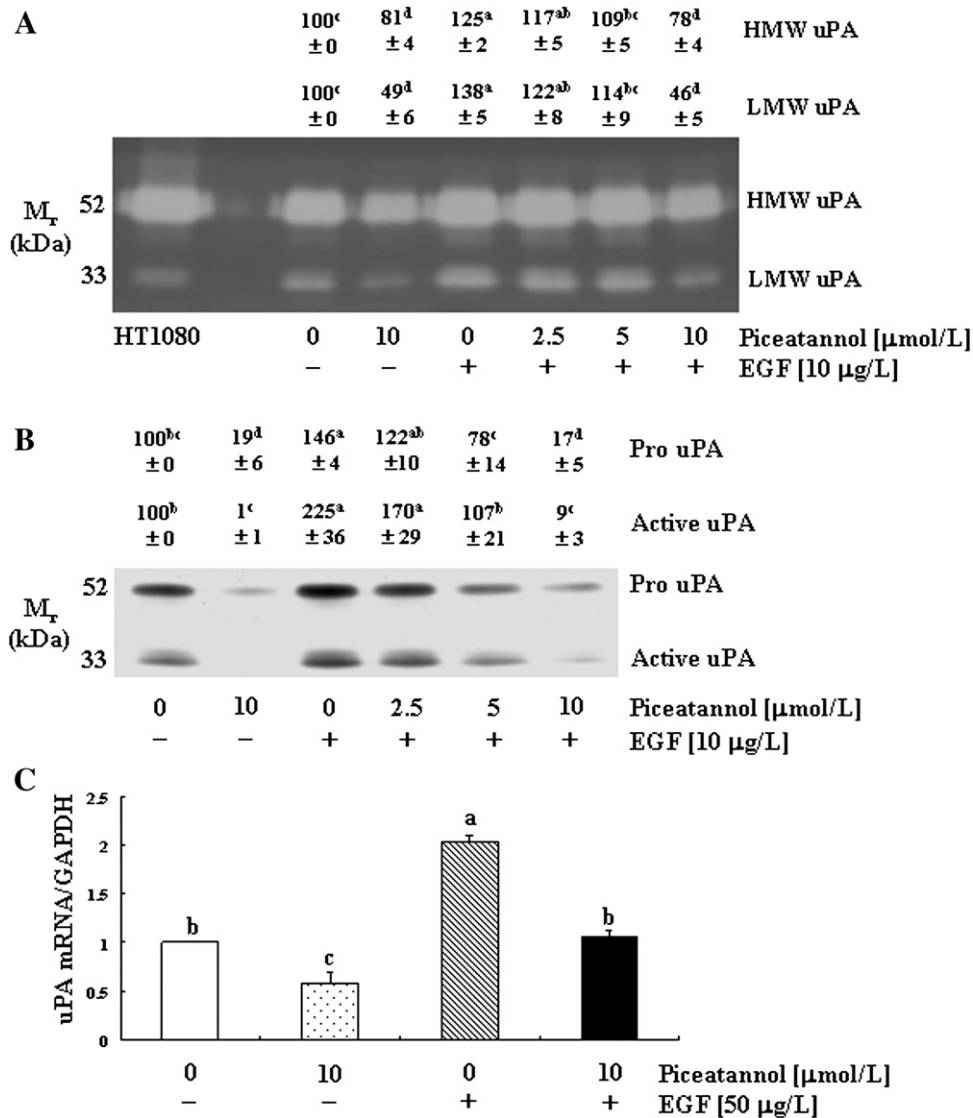


Fig. 4. Piceatannol reduces the secretion of uPA in DU145 cells. Serum-starved DU145 cells were treated with piceatannol and/or EGF as described in Fig. 3. Forty-eight-hour conditioned media were concentrated for fibrin zymography (A) and Western blotting (B). The volumes of media loaded onto the gels were adjusted for equivalent protein levels. Photographs of the Coomassie-Blue-stained gel (A) and chemiluminescent detection of the blot (B), which were representative of three independent experiments, are shown. In the first lane of (A), serum-free HT 1080 cell-conditioned medium was loaded. The relative abundance of each band was quantified, and the control levels were set at 100%. The adjusted mean \pm S.E.M. ($n=3$) of each band is shown above each blot. HMW uPA, high-molecular-weight uPA; LMW uPA, low-molecular-weight uPA. (C) Serum-starved cells were incubated with piceatannol and/or EGF for 2 h. Total RNA was isolated and reverse transcribed, and real-time PCR was conducted. The levels of uPA mRNA were normalized with those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each bar represents the mean \pm S.E.M. ($n=3$). Means without a common letter differ; $P<.05$.

were significantly reduced in the cells treated with neutralizing IL-6 antibody (Fig. 6C). Furthermore, EGF-induced cell migration was inhibited significantly by treatment with piceatannol and IL-6 neutralizing antibody (Fig. 6D).

3.5. Piceatannol inhibits IL-6-induced migration, uPA and VEGF secretion and STAT3 phosphorylation in DU145 cells

We subsequently attempted to determine whether or not exogenous IL-6 induces DU145 cell migration, via a transwell migration assay. Interleukin-6 (20 μ g/L) was shown to increase significantly the migration of DU145 cells, which was inhibited in a dose-dependent manner by piceatannol treatment (Fig. 7A). Exogenous IL-6 stimulated the secretion of pro and active forms of uPA and VEGF, and piceatannol inhibited IL-6-induced increases in the secretion of these proteins (Fig. 7B). Exogenous IL-6 treatment

induced increases in the mRNA levels of uPA and VEGF, which were suppressed by piceatannol treatment (Fig. 7C, D). The treatment of cells with exogenous IL-6 for 5 min resulted in an increase in phosphorylation on Tyr705 and Ser727 of STAT3, and piceatannol significantly inhibited these effects (Fig. 7E).

3.6. Piceatannol inhibits lung metastasis of MLL cells in nude mice

In order to determine whether piceatannol inhibits the *in vivo* metastasis of prostate cancer cells, MLL-Luc cells stably expressing firefly luciferase were injected into the tail veins of male nude mice. The bioluminescence intensity, a measure of viable MLL-Luc cells, was markedly increased 9 days after the injection of MLL-Luc cells and was significantly lower in the mice subjected to oral piceatannol administration at a dose of 20 mg/kg/d (Fig. 8).

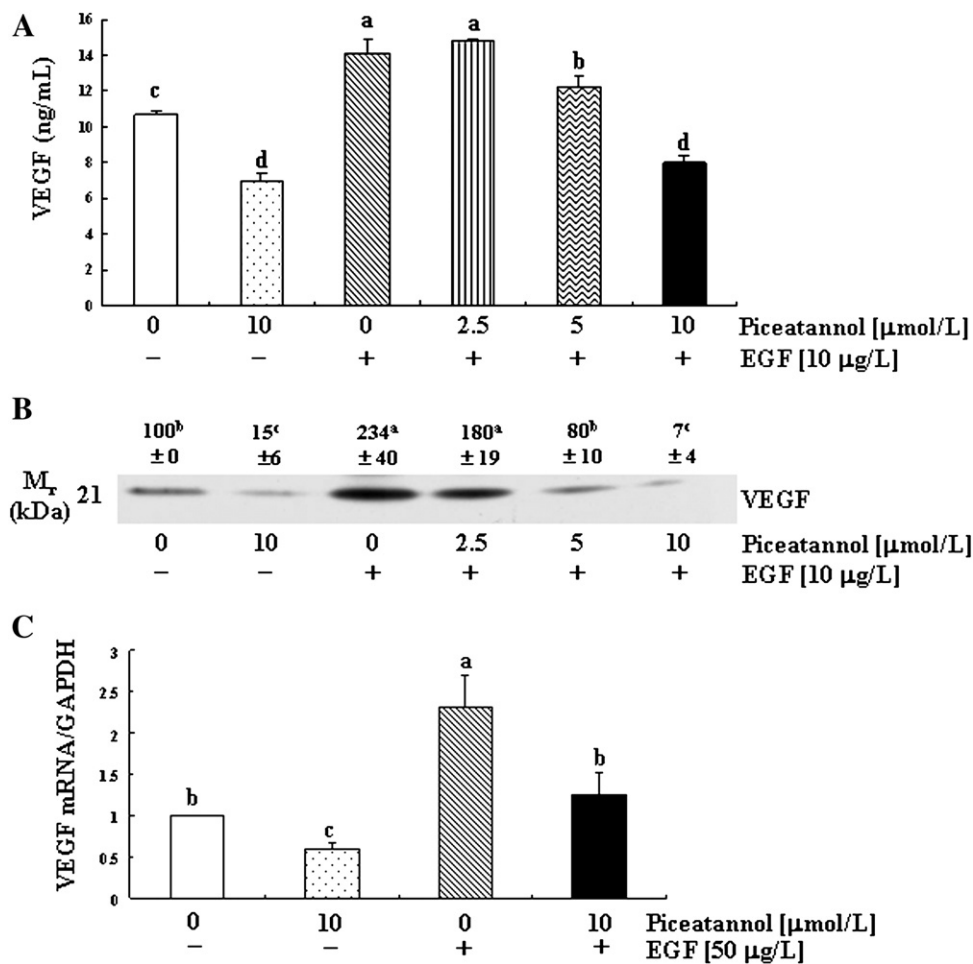


Fig. 5. Piceatannol decreases the secretion of VEGF in DU145 cells. Serum-starved DU145 cells were incubated with 0–10 $\mu\text{mol/L}$ of piceatannol in DMEM/F12 in the absence or presence of 10 $\mu\text{g/L}$ EGF. (A) Media conditioned for 24 h were collected for the VEGF ELISA assay. Each bar represents the mean \pm S.E.M. ($n=3$). (B) Media conditioned for 48 h were collected for Western blotting. A photograph of the blot, which was representative of three independent experiments, is shown. The relative abundance of each band was quantified, and the control levels were set at 100%. The adjusted mean \pm S.E.M. ($n=3$) of each band is shown above each blot. (C) Serum-starved cells were treated with piceatannol and/or EGF for 2 h. Total RNA was isolated and reverse transcribed, and real-time PCR was conducted. The levels of VEGF mRNA were normalized with those of GAPDH. Each bar represents the mean \pm S.E.M. ($n=3$).

4. Discussion

Signal transducer and activator of transcription 3 exerts oncogenic potential by promoting the expression of proteins involved in cell transformation and proliferation (c-myc, cyclin A and D, cdc25A) and down-regulating the expression of cell cycle inhibitors such as p21 and p27. It may also stimulate angiogenesis, tumor invasion and metastasis via the up-regulation of MMPs [24,25] and VEGF [26,27] expression. Signal transducer and activator of transcription 3 has been demonstrated to be constitutively activated in a broad variety of solid tumors, including prostate cancer (reviewed in Ref. [9]). Additionally, the adenoviral gene delivery of wild-type STAT3 to DU145 cells was shown to increase the number of lung metastases. The results of previous *in vitro* studies demonstrated that STAT3 promotes the migration of DU145 cells [12]. These results indicate that the bioactive compounds that interfere with STAT3 signaling may potentially be employed as agents for the inhibition of prostate cancer metastasis. Because the incidence of prostate cancer is relatively high in old men, preventing metastasis is more important than treating this disease, in terms of reducing the mortality attributable to this disease.

Piceatannol, a STAT3 inhibitor and natural polyphenol present in grapes and wine, has been shown to inhibit the lung metastasis of

Lewis lung cancer cells in C57BL/6 mice. In this animal model, the cancer cells were subcutaneously injected on day 0, the solid tumor tissue was removed on day 15, and lung metastasis was assessed on day 25 [13]. We have demonstrated previously that piceatannol (2.5–10 $\mu\text{mol/L}$) induces G1 cell cycle arrest [16] and apoptosis in DU145 cells [14]. In this study, we have demonstrated that piceatannol inhibits the migration of PC3, MLL and TRAMP-C2 prostate cancer cells. DU145 and PC3 cells were derived from metastatic human prostatic cancers. TRAMP-C2 and MLL cells were derived from a mouse and rat prostate cancer, respectively. We selected these four different types of androgen-independent prostate cancer cells because prostate cancer ultimately progresses to a hormone-refractory state, during which androgen deprivation therapy is no longer effective. Using DU145 cells, we also determined that piceatannol (a) inhibited cell migration and invasion; (b) reduced the secretion of MMP-9, uPA, VEGF and IL-6; (c) increased TIMP-2 secretion and (d) inhibited phosphorylation on Tyr705 and Ser727 of STAT3. In addition to our *in vitro* results, our *in vivo* results showed that lung metastasis could be inhibited by the oral administration of piceatannol when MLL rat prostate cancer cells stably expressing firefly luciferase were injected into the tail veins of nude mice. These results indicate that piceatannol has potential as an inhibitor of prostate cancer metastasis.

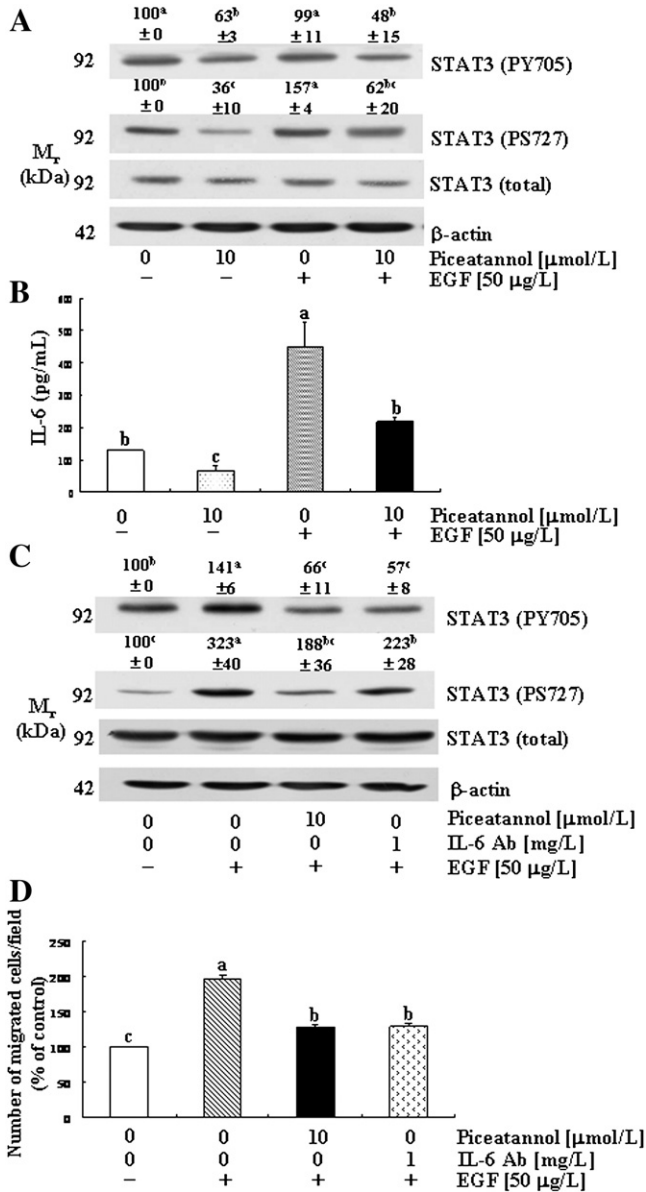


Fig. 6. Endothelial growth factor increases but piceatannol reduces IL-6 secretion in DU145 cells. DU145 cells were plated in 100-mm dishes at 1×10^6 cells/dish in DMEM/F12 supplemented with 10% FBS. One day later, the monolayers were serum starved for 24 h with serum-free DMEM/F12. (A) Cells were incubated with 0 or 10 μ mol/L of piceatannol for 2 h and lysed without stimulation (0) or after 15 min of stimulation with EGF. The cell lysates were subjected to Western blotting with indicated antibodies. (B) Cells were incubated with 0 or 10 μ mol/L piceatannol in serum-free media containing 0 or 50 μ g/L EGF for 2 h. Two-hour conditioned media were collected for IL-6 ELISA. Each bar represents the mean \pm S.E.M. ($n=3$). (C) Cells were incubated with 0 or 1 mg/L IL-6 neutralizing antibody in the absence or presence of 10 μ mol/L of piceatannol in serum-free DMEM/F12 containing 0 or 50 μ g/L EGF for 2 h. The cell lysates were subjected to Western blotting with the indicated antibodies. (A, C) Photographs of the chemiluminescent detection of the blots are shown. The relative abundance of each protein band was quantified, and the control levels were set at 100%. The adjusted mean \pm S.E.M. ($n=3$) of each band is shown above each blot. (D) The migration of DU145 cells through a type IV collagen-coated transwell filter was assessed with or without 10 μ g/L EGF, 0 or 10 μ mol/L piceatannol and/or 0 or 1 mg/L IL-6 neutralizing antibody. The cells were then incubated for 4 h. The migrated cells were quantified via the counting of H&E-stained cells. Each bar represents the mean \pm S.E.M. of three independent experiments. Means without a common letter differ; $P < .05$.

The proteolytic cleavage of extracellular matrix proteins is a critical step in cancer metastasis. Matrix metalloproteinases are a family of zinc-dependent endopeptidases that are capable of degrad-

ing ECM components. They perform important functions in tumor angiogenesis, metastasis and the release of growth factors from the ECM [28]. The activation of the uPA/plasmin proteolytic network has also been shown to play key roles in tumor invasion and the dissemination of a variety of malignancies [29,30]. In this study, the reduced secretion of MMP-2, MMP-9 (Fig. 3) and uPA (Fig. 4) and increased TIMP-2 secretion (Fig. 3B) may have contributed to the piceatannol-induced inhibition of migration and invasion in DU145 cells (Fig. 2).

In our previous study, we have noted that EGF, but not IGF-I or heregulin- β , stimulates the migration of DU145 cells [18]. Endothelial growth factor receptor (EGFR) is frequently found to be over-expressed in cases of metastatic prostate cancer [31], and its expression is correlated with disease progression and androgen-refractory diseases [32,33]. Endothelial growth factor is generated via interstitial and vascular smooth muscle cells within the human prostate [34], and EGF immunoreactive proteins are present at metastasis sites in the stroma of lymph nodes and medullary bones [35]. In this study, we noted that the EGF-induced migration and invasion of DU145 cells (Fig. 2) and the EGF-stimulated secretion of MMP-9, uPA and VEGF (Fig. 3-5) were suppressed via piceatannol treatment. Future animal studies will be required to determine whether piceatannol effectively inhibits prostate cancer metastasis involving EGFR overexpression.

The results of a previous *in vitro* study demonstrated that STAT3 can be phosphorylated directly by the EGF receptor kinase [36]. Because EGF has been reported to activate STAT3 via phosphorylation on tyrosine in EGFR-overexpressing cells [36,37], we attempted to determine whether the application of EGF treatment to the DU145 cells for 15 min induced phosphorylation on Tyr705 of STAT3. We noted that EGF induced phosphorylation on Ser727, but not on Tyr705 (Fig. 6A). It has also been reported that STAT3 is exclusively phosphorylated on Ser727 in a phosphoinositide 3-kinase- and ERK1/2-dependent manner [38]. The results of our previous study demonstrated that EGF induced Akt and ERK1/2 phosphorylation in DU145 cells [18]. Collectively, these results indicate that EGF may phosphorylate Ser727 via Akt and/or ERK1/2 activation in DU145 cells.

In order to determine whether EGF stimulates IL-6 secretion, which in turn activates phosphorylation on Tyr705 of STAT3, we incubated the cells for 2 h with EGF and measured the levels of IL-6 in the conditioned media, as well as the phosphorylation status of Tyr705. The findings that IL-6 secretion (Fig. 6B) and phosphorylation on Tyr705 (Fig. 6C) were increased in the cells treated for 2 h with EGF indicate that EGF induces phosphorylation on Tyr705 via the up-regulation of IL-6 production. As piceatannol was shown to reduce the production of IL-6 (Fig. 6B), the inhibition of STAT3 phosphorylation in piceatannol-treated cells may have been the consequence of direct inhibition by piceatannol and/or indirect inhibition resulting from reduced IL-6 production. The finding that IL-6 neutralizing antibody and piceatannol suppressed Tyr705 and Ser727 phosphorylation in cells treated with EGF for 2 h (Fig. 6C) confirms this hypothesis. It is generally acknowledged that IL-6 activates STAT3 [8] and that STAT3 regulates the expression of MMPs and VEGF [24,39]. Collectively, these results indicate that piceatannol may inhibit IL-6 production, in turn resulting in reduced STAT3 activation, ultimately leading to reduced MMP and VEGF expression in DU145 cells.

In this study, we demonstrated that IL-6 was generated by DU145 cells and that its production was increased by EGF treatment (Fig. 6B). Additionally, exogenous IL-6 induced phosphorylation on Tyr705 and Ser727 of STAT3, uPA and VEGF secretion and the migration of DU145 cells (Fig. 7). Moreover, the IL-6 neutralizing antibody was shown to suppress EGF-induced STAT3 activation (Fig. 6C) and the migration of these cancer cells (Fig. 6D). These results clearly indicate that IL-6 is

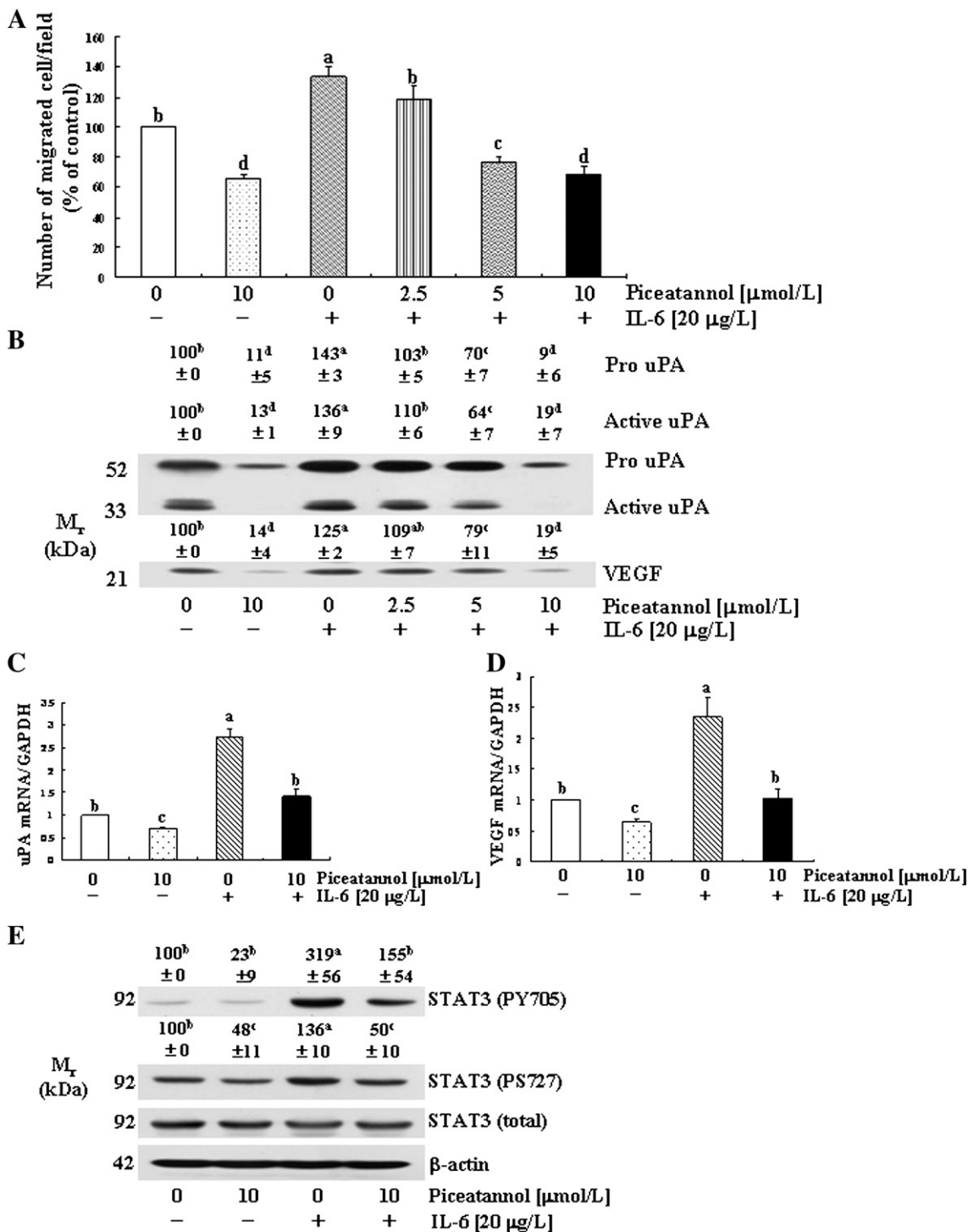


Fig. 7. Effect of piceatannol on IL-6-induced secretion of uPA and VEGF in DU145 cells. (A) The migration of DU145 cells through a type IV collagen-coated transwell filter was assessed with 0–10 $\mu\text{mol/L}$ piceatannol, with or without 20 $\mu\text{g/L}$ IL-6. Cells were incubated for 4 h. (B) Serum-starved DU145 cells were incubated with 0–10 $\mu\text{mol/L}$ of piceatannol in DMEM/F12 in the presence or absence of 20 $\mu\text{g/L}$ IL-6 for 12 h. Media conditioned for 12 h were collected and concentrated, and Western blotting was conducted. The volumes of media loaded onto the gel were adjusted for equivalent protein levels. (C, D) Serum-starved cells were incubated for 2 h with piceatannol and/or IL-6. Total RNA was isolated and reverse transcribed, and real-time PCR was conducted. (E) Serum-starved cells were incubated for 1 h in serum-free DMEM/F12 in the presence or absence of 10 $\mu\text{mol/L}$ of piceatannol and lysed without stimulation (0) or after 5 min of stimulation with 20 $\mu\text{g/L}$ of recombinant IL-6 protein. Cell lysates were subjected to Western blotting with indicated antibodies. Photographs of the chemiluminescent detection of the blots are shown. The relative abundance of each protein band was quantified, and the control levels were set at 100%. The adjusted mean \pm S.E.M. ($n=3$) of each band is shown above each blot. Means without a common letter differ; $P<.05$.

an autocrine factor that stimulates the metastatic potential of DU145 cells. These results also demonstrate that the stimulatory effects of EGF on the migration and invasion of DU145 cells were mediated, at least in part, by the up-regulation of IL-6 production.

Piceatannol inhibited phosphorylation on Tyr705 and Ser727 of STAT3 (Fig. 6A), along with a reduction in IL-6 secretion (Fig. 6B). Because piceatannol is a known inhibitor of STAT3 [5] and IL-6 can be up-regulated by STAT3 (reviewed in Ref. [40]), it may be surmised

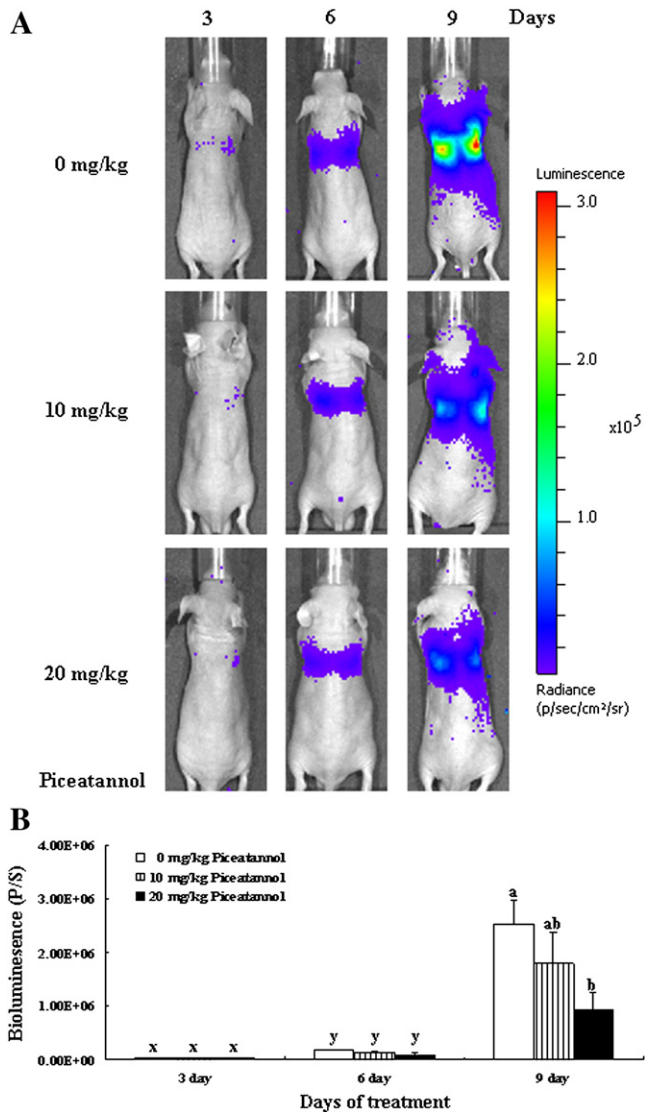


Fig. 8. Piceatannol suppresses the lung metastasis of MLL-Luc cells in nude mice. MLL-Luc cells were injected into the tail veins of male nude mice. The mice were subjected to daily oral gavage with piceatannol at doses of 0, 10 or 20 mg/kg/d, commencing 1 day after the MLL-Luc injection. Bioluminescence imaging (BLI) was conducted at 3, 6 and 9 days. (A) Representative BLI in mice, which reveals the progression of lung metastasis at different time points after MLL-Luc injection, is shown. The scale on the right of the image indicates the surface radiance (photons/s/cm²/steradian). (B) The signal intensity was quantified as the sum of all detected photon counts/s in the region of interest (mean ± S.E.M., n=6). Means without a common letter differ; *P*<.05.

that piceatannol inhibits the activation of STAT3, which in turn inhibits IL-6 expression in DU145 cells. Additionally, as IL-6 activates STAT3 [10,11], this reduced IL-6 expression may have induced an additional reduction in IL-6 expression via the reduction of STAT3 activation in piceatannol-treated cells.

To the best of our knowledge, no data are currently available regarding equivalent doses of piceatannol in humans. The Food and Drug Administration (FDA) [41] has suggested that the human dose can be extrapolated from the animal data via normalization to the body surface area (BSA). To convert the doses (10–20 mg/kg) of piceatannol used in mice to a human equivalent dose, we can use the following formula by Reagan-Shaw et al. [42], which is derived from the FDA Draft guidelines: human equivalent dose (mg/kg)=mouse dose (mg/kg)×mouse[weight (kg)/BSA]/human [weight (kg)/BSA]. This calculation results in human equivalent doses for piceatannol of

0.81–1.62 mg/kg, which equates to a 49–97-mg dose of piceatannol for a 60-kg human. More studies will be necessary to translate the dosage of piceatannol from animal studies to human studies.

In conclusion, this study demonstrates that piceatannol reduces the migration and invasion characteristics of androgen-independent DU145 prostate cancer cells, which may be mediated via the inhibition of MMP-9 and uPA activities and VEGF secretion. The inhibition of IL-6 secretion may be one of the mechanisms by which piceatannol inhibits STAT3 activation, which in turn reduces the expression of MMP-9 and VEGF in DU145 cells. Our results indicate that EGF induces the secretion of IL-6, which in turn activates STAT3, resulting in the up-regulation of MMP-9 and VEGF as well as the migration and invasion of DU145 cells. We also demonstrated that piceatannol inhibits the lung metastasis of prostate cancer cells in mice. Because the incidence of prostate cancer is relatively high in elderly men and hormone-refractory prostate cancer does not respond well to the currently available chemotherapeutic agents, the piceatannol-induced inhibition of metastatic potential in androgen-independent prostate cancer cells may generate significant preventive benefits.

Supplementary materials related to this article can be found online at doi:10.1016/j.jnutbio.2010.11.019.

References

- [1] Sporn MB, Suh N. Chemoprevention of cancer. *Carcinogenesis* 2000;21:525–30.
- [2] Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. *CA Cancer J Clin* 2009;59:225–49.
- [3] Kasamon KM, Dawson NA. Update on hormone-refractory prostate cancer. *Curr Opin Urol* 2004;14:185–93.
- [4] Roupe KA, Remsburg CM, Yanez JA, Davies NM. Pharmacometrics of stilbenes: segueing towards the clinic. *Curr Clin Pharmacol* 2006;1:81–101.
- [5] Su L, David M. Distinct mechanisms of STAT phosphorylation via the interferon- α /beta receptor. Selective inhibition of STAT3 and STAT5 by piceatannol. *J Biol Chem* 2000;275:12661–6.
- [6] Geahlen RL, McLaughlin JL. Piceatannol (3,4,3',5'-tetrahydroxy-trans-stilbene) is a naturally occurring protein-tyrosine kinase inhibitor. *Biochem Biophys Res Commun* 1989;165:241–5.
- [7] Levy DE, Darnell Jr JE. Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol* 2002;3:651–62.
- [8] Yu H, Jove R. The STATs of cancer – new molecular targets come of age. *Nat Rev Cancer* 2004;4:97–105.
- [9] Calo V, Migliavacca M, Bazan V, Macaluso M, Buscemi M, Gebbia N, Russo A. STAT proteins: from normal control of cellular events to tumorigenesis. *J Cell Physiol* 2003;197:157–68.
- [10] Selander KS, Li L, Watson L, Merrell M, Dahmen H, Heinrich PC, Muller-Newen G, Harris KW. Inhibition of gp130 signaling in breast cancer blocks constitutive activation of Stat3 and inhibits in vivo malignancy. *Cancer Res* 2004;64:6924–33.
- [11] Buettner R, Mora LB, Jove R. Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. *Clin Cancer Res* 2002;8:945–54.
- [12] Abdulghani J, Gu L, Dagvadorj A, Lutz J, Leiby B, Bonucelli G, Lisanti MP, Zellweger T, Alanen K, Mirtti T, Visakorpi T, Bubendorf L, Nevalainen MT. Stat3 promotes metastatic progression of prostate cancer. *Am J Pathol* 2008;172:1717–28.
- [13] Kimura Y, Baba K, Okuda H. Inhibitory effects of active substances isolated from *Cassia garrettiana* heartwood on tumor growth and lung metastasis in Lewis lung carcinoma-bearing mice (Part 2). *Anticancer Res* 2000;20:2923–30.
- [14] Kim EJ, Park H, Park SY, Jun JG, Park JH. The grape component piceatannol induces apoptosis in DU145 human prostate cancer cells via the activation of extrinsic and intrinsic pathways. *J Med Food* 2009;12:943–51.
- [15] Barton BE, Karras JG, Murphy TF, Barton A, Huang HF. Signal transducer and activator of transcription 3 (STAT3) activation in prostate cancer: direct STAT3 inhibition induces apoptosis in prostate cancer lines. *Mol Cancer Ther* 2004;3:11–20.
- [16] Lee YM, Lim do Y, Cho HJ, Seon MR, Kim JK, Lee BY, Park JH. Piceatannol, a natural stilbene from grapes, induces G1 cell cycle arrest in androgen-insensitive DU145 human prostate cancer cells via the inhibition of CDK activity. *Cancer Lett* 2009;285:166–73.
- [17] Kim EJ, Holthuizen PE, Park HS, Ha YL, Jung KC, Park JHY. *Trans-10,cis-12* conjugated linoleic acid inhibits Caco-2 colon cancer cell growth. *Am J Physiol Gastrointest Liver Physiol* 2002;283:G357–67.
- [18] Kwon GT, Cho HJ, Chung WY, Park KK, Moon A, Park JH. Isoliquiritigenin inhibits migration and invasion of prostate cancer cells: possible mediation by decreased JNK/AP-1 signaling. *J Nutr Biochem* 2009;20:663–76.

- [19] Yodkeeree S, Garbisa S, Limtrakul P. Tetrahydrocurcumin inhibits HT1080 cell migration and invasion via downregulation of MMPs and uPA. *Acta Pharmacol Sin* 2008;29:853–60.
- [20] Moll UM, Youngleib GL, Rosinski KB, Quigley JP. Tumor promoter-stimulated Mr 92,000 gelatinase secreted by normal and malignant human cells: isolation and characterization of the enzyme from HT1080 tumor cells. *Cancer Res* 1990;50:6162–70.
- [21] Azzam HS, Thompson EW. Collagen-induced activation of the M(r) 72,000 type IV collagenase in normal and malignant human fibroblastoid cells. *Cancer Res* 1992;52:4540–4.
- [22] Cho HJ, Kim WK, Kim EJ, Jung KC, Park S, Lee HS, Tyner AL, Park JH. Conjugated linoleic acid inhibits cell proliferation and ErbB3 signaling in HT-29 human colon cell line. *Am J Physiol Gastrointest Liver Physiol* 2003;284:G996–1005.
- [23] Kim EJ, Kang IJ, Cho HJ, Kim WK, Ha YL, Park JH. Conjugated linoleic acid downregulates insulin-like growth factor-I receptor levels in HT-29 human colon cancer cells. *J Nutr* 2003;133:2675–81.
- [24] Xie TX, Wei D, Liu M, Gao AC, Ali-Osman F, Sawaya R, Huang S. Stat3 activation regulates the expression of matrix metalloproteinase-2 and tumor invasion and metastasis. *Oncogene* 2004;23:3550–60.
- [25] Dechow TN, Pedranzini L, Leitch A, Leslie K, Gerald WL, Linkov I, Bromberg JF. Requirement of matrix metalloproteinase-9 for the transformation of human mammary epithelial cells by Stat3-C. *Proc Natl Acad Sci U S A* 2004;101:10602–7.
- [26] Niu G, Wright KL, Huang M, Song L, Haura E, Turkson J, Zhang S, Wang T, Sinibaldi D, Coppola D, Heller R, Ellis LM, Karras J, Bromberg J, Pardoll D, Jove R, Yu H. Constitutive Stat3 activity up-regulates VEGF expression and tumor angiogenesis. *Oncogene* 2002;21:2000–8.
- [27] Alvarez JV, Febbo PG, Ramaswamy S, Loda M, Richardson A, Frank DA. Identification of a genetic signature of activated signal transducer and activator of transcription 3 in human tumors. *Cancer Res* 2005;65:5054–62.
- [28] Coussens LM, Werb Z. Matrix metalloproteinases and the development of cancer. *Chem Biol* 1996;3:895–904.
- [29] Andreasen PA, Kjoller L, Christensen L, Duffy MJ. The urokinase-type plasminogen activator system in cancer metastasis: a review. *Int J Cancer* 1997;72:1–22.
- [30] Kwaan HC. The plasminogen-plasmin system in malignancy. *Cancer Metastasis Rev* 1992;11:291–311.
- [31] Zellweger T, Ninck C, Bloch M, Mirlacher M, Koivisto PA, Helin HJ, Mihatsch MJ, Gasser TC, Bubendorf L. Expression patterns of potential therapeutic targets in prostate cancer. *Int J Cancer* 2005;113:619–28.
- [32] Di Lorenzo G, Tortora G, D'Armiento FP, De Rosa G, Staibano S, Autorino R, D'Armiento M, De Laurentiis M, De Placido S, Catalano G, Bianco AR, Ciardiello F. Expression of epidermal growth factor receptor correlates with disease relapse and progression to androgen-independence in human prostate cancer. *Clin Cancer Res* 2002;8:3438–44.
- [33] Hernes E, Fossa SD, Berner A, Otnes B, Nesland JM. Expression of the epidermal growth factor receptor family in prostate carcinoma before and during androgen-independence. *Br J Cancer* 2004;90:449–54.
- [34] Freeman MR, Paul S, Kaefer M, Ishikawa M, Adam RM, Renshaw AA, Elenius K, Klagsbrun M. Heparin-binding EGF-like growth factor in the human prostate: synthesis predominantly by interstitial and vascular smooth muscle cells and action as a carcinoma cell mitogen. *J Cell Biochem* 1998;68:328–38.
- [35] Rajan R, Vanderslice R, Kapur S, Lynch J, Thompson R, Djakiew D. Epidermal growth factor (EGF) promotes chemomigration of a human prostate tumor cell line, and EGF immunoreactive proteins are present at sites of metastasis in the stroma of lymph nodes and medullary bone. *Prostate* 1996;28:1–9.
- [36] Park OK, Schaefer TS, Nathans D. In vitro activation of Stat3 by epidermal growth factor receptor kinase. *Proc Natl Acad Sci U S A* 1996;93:13704–8.
- [37] Zhong Z, Wen Z, Darnell Jr JE. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science* 1994;264:95–8.
- [38] Su L, Rickert RC, David M. Rapid STAT phosphorylation via the B cell receptor. Modulatory role of CD19. *J Biol Chem* 1999;274:31770–4.
- [39] Loeffler S, Fayard B, Weis J, Weissenberger J. Interleukin-6 induces transcriptional activation of vascular endothelial growth factor (VEGF) in astrocytes in vivo and regulates VEGF promoter activity in glioblastoma cells via direct interaction between STAT3 and Sp1. *Int J Cancer* 2005;115:202–13.
- [40] Yu H, Kortylewski M, Pardoll D. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. *Nat Rev Immunol* 2007;7:41–51.
- [41] U.S. Food and Drug Administration. Estimating the safe starting dose in clinical trials for therapeutics in adult healthy volunteers. Rockville, MD: Center for Drug Evaluation and Research and Center for Biologics Evaluation and Research; 2002.
- [42] Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. *Faseb J* 2008;22:659–61.