RESEARCH ARTICLE

Suppression of EGF-induced tumor cell migration and matrix metalloproteinase-9 expression by capsaicin via the inhibition of EGFR-mediated FAK/Akt, PKC/Raf/ERK, p38 MAPK, and AP-1 signaling

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Scope: Capsaicin is a cancer-suppressing agent. The aim of our study was to determine the effect of capsaicin on tumor invasion and migration; the possible mechanisms involved in this inhibition were investigated in human fibrosarcoma cells.

Methods and results: We employed invasion, migration and gelatin zymography assays to characterize the effect of capsaicin on HT-1080 cells. Transient transfection assays and immunoblot analysis were performed to study its molecular mechanisms of action. Capsaicin inhibited the epidermal growth factor (EGF)-induced activation of matrix metalloproteinase (MMP)-9 and MMP-2, and further inhibited cell invasion and migration. Capsaicin decreased the EGF-induced expression of MMP-9, MMP-2, and MT1-MMP, but did not alter TIMP-1 and TIMP-2 levels. Capsaicin suppressed EGF-induced c-Jun and c-Fos nuclear translocation, and also abrogated the EGF-induced phosphorylation of EGF receptor (EGFR), focal adhesion kinase (FAK), protein kinase C (PKC), phosphatidylinositol 3-Kinase (PI3K)/Akt, extracellular regulated kinase (ERK)1/2, and JNK1/2, an upstream modulator of AP-1. Furthermore, the EGFR inhibitor inhibited EGF-induced MMP-9 expression, as well as AP-1 activity and cell migration.

Conclusion: Capsaicin inhibited the EGF-induced invasion and migration of human fibrosarcoma cells via EGFR-dependent FAK/Akt, PKC/Raf/ERK, p38 mitogen-activated protein kinase (MAPK), and AP-1 signaling, leading to the down-regulation of MMP-9 expression. These results indicate the role of capsaicin as a potent anti-metastatic agent, which can markedly inhibit the metastatic and invasive capacity of fibrosarcoma cells.

Keywords:
Anti-metastatic / AP-1 / Capsaicin / EGF / MMP-9

1 Introduction

Tumor cell invasion and migration involve the proteolytic degradation of extracellular matrix (ECM) components by tumor cell-secreted proteases, including serine proteases, plasminogen activators, and matrix metalloproteinases (MMPs) [1, 2]. Many tumors exhibit elevated levels of MMPs, which may play an important role in cellular invasion and metastasis [3]. Among the MMPs identified, MMP-9 (gelatinase B) functions in many physiological and

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Abbreviations: ECM, extracellular matrix; **EGF,** epidermal growth factor; **EGFR,** EGF receptor; **EMSA,** electrophoretic mobility shift assay; **ERK,** extracellular regulated kinase; **FAK,** focal adhesion kinase; **FBS,** fetal bovine serum; **GAPDH,** glyceraldehyde phosphate dehydrogenase; **LDH,** lactate dehydrogenase; **MAPK,** mitogen-activated protein kinase; **MMPs,** matrix metalloproteinases; **PI3K,** phosphatidylinositol 3-kinase; **PKC,** protein kinase C; **Ras-MAPK,** Ras-mitogen-activated protein kinase; **WST,** water-soluble tetrazolium

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pathophysiological processes, including placental development, wound healing, angiogenesis, inflammation, and tumor invasion/metastasis [4]. MMPs are synthesized as pre-proenzymes and are secreted from cells as proenzymes. Among the human MMPs reported to date, MMP-2 and MMP-9 are the major enzymes involved in degrading types I and IV collagen and the ECM [5]. The MMP family of zinc-dependent proteases is divided into four subclasses based on their substrate specificity: collagenase, gelatinase, stromelysin, and membrane-associated MMPs [6]. Tumor-secreted MMPs destroy the ECM components in tissues surrounding a tumor, enabling tumor cells to pass through the basement membrane of blood vessels and facilitating their spread to distant organs, resulting in organ failure and patient mortality. MMP-2 and MMP-9, which are abundantly expressed in various malignant tumors, contribute to cancer invasion and metastasis [5]. Generally, MMP-2 is constitutively overexpressed in highly metastatic tumors, whereas MMP-9 can be stimulated by an inflammatory cytokine, epidermal growth factor (EGF), or the phorbol ester 12-O-tetradecanoylphorbol-13-acetate, through the activation of different intracellular signaling pathways [7, 8].

EGF-induced MMP-9 secretion, which facilitates tumor invasion and metastasis, has been demonstrated in various tumor cells [9, 10]. Dysregulation of human EGF receptor (EGFR) signaling due to overexpression or constitutive activation can promote tumor processes, including angiogenesis and metastasis, and is associated with a poor prognosis in many human malignancies [11, 12]. In various systems, EGFR ligation leads to the activation of focal adhesion kinase (FAK), phosphatidylinositol 3-kinase (PI3K), signal transducer and activator of transcription (STAT), phospholipase C-protein kinase C (PLC-PKC), and Ras-mitogen-activated protein kinase (Ras-MAPK) signaling [13, 14], resulting in the activation of several transcription factors. Each of these pathways has been implicated in growth control and survival. In addition, the EGFR-mediated activation of PLC and MAPKs has been linked to migration and invasion [15, 16]. The potential value of modulating EGFR signaling as a cancer treatment approach is reflected by the various molecular inhibitors that have been developed and launched in clinical trials in recent years. The molecular inhibition of EGFR signaling is under active investigation as a promising cancer treatment strategy [17].

Cancer chemoprevention has gained considerable attention recently, probably because it involves the use of dietary bioactive compounds, either alone or in combination, to reverse, suppress, or prevent cancer progression [18]. Epidemiological and laboratory studies have demonstrated that dietary agents are important factors in reducing cancer risk [19, 20]. Importantly, most of these bioactive substances exert their anti-cancer properties by reducing MMP activation and triggering tumor cell invasion and migration.

Capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) is the main active ingredient and spicy component of hot chili peppers (genus *Capsicum*), which are among the most commonly and frequently used spices worldwide [21]. Due to its analgesic and anti-inflammatory activities, the topical application of capsaicin has been used in clinical practice for the treatment of various neuropathic conditions, including cluster headaches, diabetic neuropathy, and rheumatoid arthritis [22, 23]. Capsaicin was found to repress the growth of various immortalized or malignant cell lines through the induction of apoptosis and the inhibition of angiogenesis [24–26]. Moreover, a recent study revealed the ability of capsaicin to inhibit the *in vitro* and *in vivo* migration of malignant glioma cells [25, 27], indicating its potential usefulness in anti-cancer therapy. Although various bioactivity studies of capsaicin have been performed, the molecular mechanisms by which capsaicin affects the expression of MMP-9 and the invasiveness of HT-1080 cells remain unclear. In this study, we examined the effects of capsaicin on EGF-induced MMP-9 and MMP-2 expressions, and explored the underlying upstream molecular signaling events.

2 Materials and methods

2.1 Materials

AG 1478 (AG), Rottlerin (RO), Gö 6976 (Gö), LY 294002 (LY), PD 98059 (PD), and SB 203580 (SB) were obtained from Calbiochem (La Jolla, CA, USA). Capsaicin, curcumin, and EGF were purchased from Sigma Chemical (St. Louis, MO, USA). Water-soluble tetrazolium (WST)-1 and lactate dehydrogenase (LDH) assay kits were purchased from Roche Diagnostics GmbH (Mannheim, Germany). RPMI1640, fetal bovine serum (FBS), sodium pyruvate, and Trizol reagent were supplied by Gibco BRL (Grand Island, NY, USA). Antibodies against phospho-EGFR, phospho-FAK, phospho-Akt, phospho-Raf, phospho-MAPK, phospho-PKC, MMP-2, MMP-9, and NF-κB were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against c-Jun, c-Fos, lamin B, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The *MMP-9* promoter vector was kindly provided by Dr. W. Eberhardt (Klinikum der Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany) [28]. The pNF-κB-Luc and pAP-1-Luc reporter plasmids were obtained from Stratagene (La Jolla, CA, USA). All other chemicals and reagents were of analytical grade.

2.2 Cell culture and measurement of cell viability

HT-1080 cells from the ATCC (American Type Culture Collection, Manassas, VA, USA) were grown in RPMI1640 supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO_2 humidified incubator. The cells were then treated with different concentrations of capsaicin in the absence or presence of 100 ng/mL EGF for 24 h. Capsaicin was prepared as

an ethanol stock solution, which was further diluted in intracellular solution prior to use. The cytotoxic effect of capsaicin on HT-1080 cells was investigated using a commercially available proliferation kit (WST1 and LDH assay, Roche Diagnostics GmbH, Mannheim, Germany) as described previously [29]. Cells were plated in a 96-well culture plate at a density of 5×10^3cells/well.

2.3 Wound-healing assay

HT-1080 cells were seeded in a six-well plate and grown overnight to confluence. The monolayers were scratched with a 200-μL pipette tip to create a wound, and then washed twice with serum-free RPMI1640 to remove floating cells. The medium was then replaced with serum-free medium. The rate of closure was assessed, and the wound was photographed 24 h later. Each value is derived from three randomly selected fields.

2.4 Matrigel invasion assay

HT-1080 cells were incubated in RPMI1640 with 10% FBS and then collected by trypsinization. Cells (1×10^5 cells/mL) in serum-free medium were added to the inner cup of a 48-well Transwell chamber (Corning Life Sciences, Oneonta, NY, USA) that had been coated with 50 μL of matrigel (BD Biosciences, Franklin Lakes, NJ, USA; 1:10 dilution in serum-free medium). Medium was supplemented with 10% serum or the indicated agent was added to the outer cup. After 24 h, cells that had migrated through the matrigel and the 8-μm pore size membrane were fixed, stained, and counted under a light microscope. Each experiment was performed in triplicate.

2.5 RNA preparation and semi-quantitative PCR

Total RNA was isolated with an RNA extraction kit (Amersham Pharmacia Biotech, Buckinghamshire, UK), and the total RNA concentration was measured using a spectrophotometer. RNA (2 μg) was converted to cDNA using a reverse transcriptase-polymerase chain reaction (RT-PCR) Bead kit (Amersham Pharmacia Biotech) according to the manufacturer's protocol. The PCR amplification protocol was 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. The products were resolved by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light.

2.6 Real-time PCR

PCR product formation was monitored continuously during the reaction using Sequence Detection System software, version 1.7 (Applied Biosystems, Foster City, CA, USA). Accumulated PCR products were detected directly by monitoring the increase in SYBR reporter dye (Invitrogen, Carlsbad, CA, USA). The mRNA expression levels of *MMP-2* and *MMP-9* in the treated cells were compared to the expression levels in control cells at each time point using the comparative cycle threshold (Ct)-method [30]. The following primers were used in this study: MMP-2 forward, 5′-AGTCTGAAGA GCGTGAAG-3′ and MMP-2 reverse, 5′-CCAGGTAGGAGT GAGAATG-3′; MMP-9 forward, 5′-TGACAGCG ACAAGAAGTG-3′ and MMP-9 reverse, 5′-CAGTGAAGCGG TACATAGG-3′; glyceraldehyde phosphate dehydrogenase (GAPDH) forward, 5′-CCACCCATGGCAAATTCC-3′ and GAPDH reverse, and 5′-TGGGATTTCCATTGATGACAA-3′. The quantity of each transcript was calculated as described in the instrument manual and normalized to the expression of *GAPDH*, a housekeeping gene.

2.7 Western-blot analysis

After treatment, cells were collected and washed with phosphate-buffered saline (PBS). The harvested cells were then lysed on ice for 30 min in 100 μL of lysis buffer (120 mM NaCl, 40 mM Tris [pH 8], and 0.1% NP40) and centrifuged at $13\,000 \times g$ for 15 min. The supernatants were collected and protein concentrations were determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Then to determine the proteins levels associated with invasion and migration. All samples were separated by SDS-PAGE, as described previously [31].

2.8 Gelatin zymography

The gelatinolytic activity of MMP-9 and MMP-2 secreted in conditioned media was assayed by means of gelatin-substrate gel electrophoresis as described previously [31]. Gelatinolytic activity was normalized against the protein content of the cultured cells as assayed using a BCA kit (Pierce Biotechnology).

2.9 Transient transfection and luciferase assay

To assess promoter activity, we used a dual-luciferase reporter assay system (Promega, Madison, WI, USA). Cells were plated in 48-well plates and incubated at 37°C. At 70–80% confluence, the cells were washed with RPMI1640 and incubated with RPMI1640 without serum or antibiotics for 6 h. The *MMP-9* promoter vector, AP-1, or NF-B reporter vector (1 μg each; Stratagene) and pCMV-β-gal (0.5 μg) plasmids were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. After incubation, the cells were lysed and luciferase activity was measured using a luminometer (Luminoscan Ascent; Thermo Electron, Berthold, Germany).

Luciferase activity was normalized to β-galactosidase activity in the cell lysates and expressed as an average of three independent experiments. For the analysis of basal AP-1 activity, cells were transiently transfected with Lenti-AP-1-GFP (System Biosciences, Mountain View, CA, USA), and then mixed with the Lipofectamine reagent. GFP fluorescence was analyzed by fluorescence microscopy (Axiovert 200M; Carl Zeiss, Jena, Germany).

2.10 Electrophoretic mobility shift assay (EMSA)

HT-1080 cells were treated with EGF in the absence or presence of capsaicin, and nuclear extracts were prepared as described previously [32]. Nuclear proteins (5 μg) were incubated with ^{32}P-labeled AP-1 probe (5′-CGCTTGAT-GAGTCAGCCGGAAC-3′) on ice for 30 min, and the DNA-protein complex was separated on a 5% acrylamide gel.

2.11 Statistical analyses

All experiments were repeated at least three times. Means ± SD were calculated for each group, and Dunnett's *t*-test was used to calculate statistical significance. Differences were considered statistically significant at $p < 0.01$.

3 Results

3.1 Capsaicin inhibits the invasion and migration of human fibrosarcoma cells

Prior to investigating the pharmacological potential of capsaicin on EGF-induced MMP activity, we first determined the dose dependence of the cytotoxic effects of capsaicin in the absence or presence of EGF (100 ng/mL) for 24 h in HT-1080 cells using the WST-1 and LDH assays. As the viability of HT-1080 cells was not significantly affected by capsaicin at concentrations up to 50 μM in the absence or presence of EGF (Fig. 1A and B), we chose a concentration of 10–50 μM capsaicin for our subsequent experiments. Next, we cultured cells in the presence of EGF to examine whether capsaicin inhibits HT-1080 cell invasion and migration (including Transwell and wound-healing assays). Transwell (Fig. 1C) migration assays revealed that capsaicin significantly inhibited EGF-induced HT-1080 cell invasion.

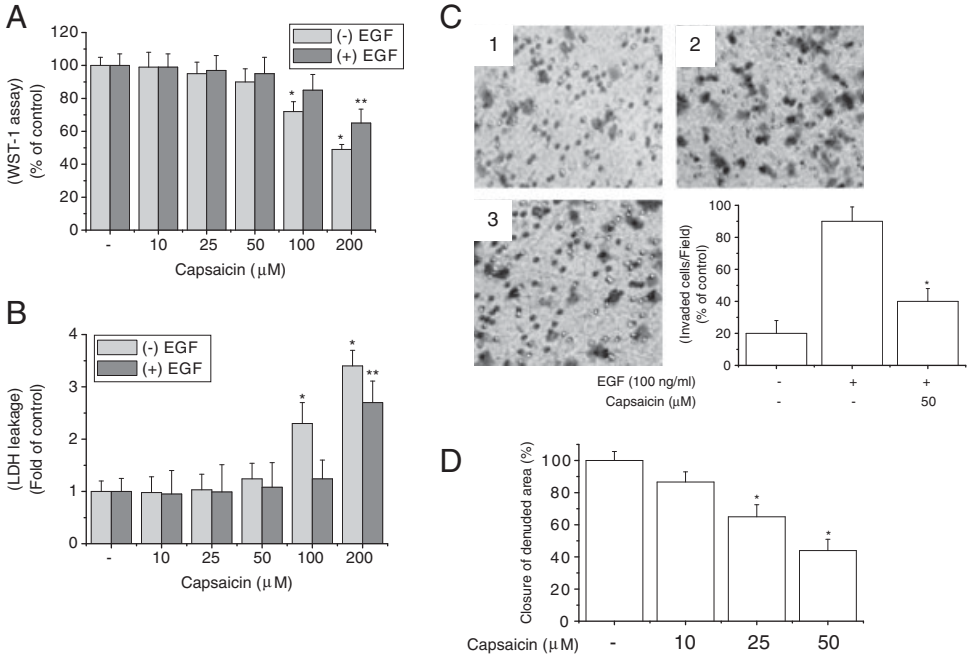

Figure 1. Inhibitory effects of capsaicin on migration and invasion in human HT-1080 fibrosarcoma cells. (A and B) Effect of capsaicin on cell cytotoxicity. HT-1080 cells were treated with capsaicin (0, 10, 25, 50, 100, and 200 μM) in the absence or presence of 100 ng/mL EGF for 24 h in serum-free medium, and viability was determined by the WST-1 (A) and LDH release assays (B). *Significantly different from the control ($p < 0.01$). **Significantly different from EGF treatment only ($p < 0.01$). (C) Effects of capsaicin on invasion in HT-1080 cells. Cells were pretreated with capsaicin (50 μM) followed by EGF (100 ng/mL) treatment for 24 h. After 24 h, cells on the bottom side of the filter were counted. (1) Control; (2) EGF alone; (3) EGF with capsaicin (50 μM). *Significantly different from EGF treatment only ($p < 0.01$). (D) Effects of capsaicin on migration in HT-1080 cells. Cells were scratched with a pipette tip and then treated with capsaicin (10, 25, and 50 μM) for 24 h. Migrating cells were photographed under phase-contrast microscopy. Each bar represents the mean ± SD calculated from three independent experiments. *Significantly different from the control ($p < 0.01$).

Also, HT-1080 cell migration was inhibited by capsaicin (Fig. 1D). These results suggest that capsaicin prevents the invasion and migration of human fibrosarcoma cells at non-toxic concentrations.

3.2 Capsaicin suppresses EGF-induced MMP-9 and MMP-2 expressions

Since the proteolytic cleavage of ECM proteins is a necessary step in the invasion of cancer cells, we next examined the effect of capsaicin on MMP activity, which is related to the invasion and metastasis of fibrosarcomas. Capsaicin inhibited EGF-induced MMP-9 and MMP-2 activity in a dose-dependent manner, as demonstrated by gelatin zymography and Western-blot analysis (Fig. 2A). To determine whether the inhibition of MMP-9 secretion by capsaicin was due to decreased transcription, we performed RT-PCR and promoter assays using transiently transfected cells with a *luciferase* reporter gene linked to the *MMP-9* promoter sequence. As shown by semi-quantitative RT-PCR and real-time PCR, the treatment of HT-1080 cells with capsaicin decreased the level of EGF-stimulated *MMP-9* and *MMP-2* mRNA expressions (Fig. 2B–D), indicating that capsaicin decreased the transcription of *MMP-9* and *MMP-2* in response to EGF. The effect of capsaicin on *MMP-9* promoter activity was also investigated using HT-1080 cells that had been transiently transfected with a *luciferase* reporter gene linked to the *MMP-9* promoter sequence. As shown in Fig. 2E, treatment with capsaicin (10–50 μM) decreased EGF-mediated luciferase activity in a dose-dependent manner, indicating that capsaicin inhibited *MMP-9* expression at the transcriptional level and

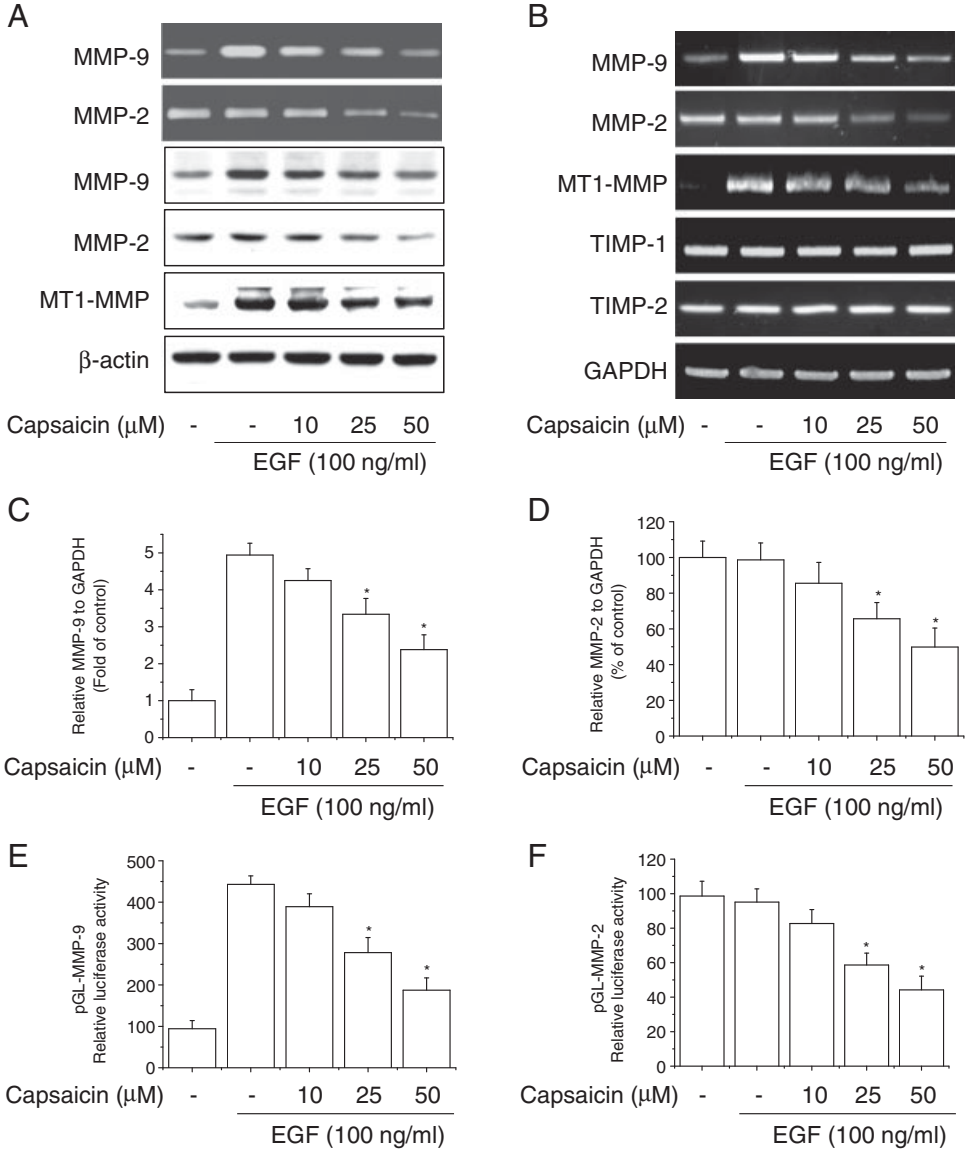

Figure 2. Inhibition of EGF-induced MMP-9 and MMP-2 expression by capsaicin. (A) Effects of capsaicin on MMP-9 activity in HT-1080 cells. Cells were treated with various concentrations of capsaicin (10, 25, and 50 μM) for 24 h in the presence of EGF (100 ng/mL). MMP-9, MMP-2 and MT1-MMP expressions in HT-1080 cells were determined by gelatin zymography and Western-blot analysis. (B and D) Effects of capsaicin on *MMP-9, MMP-2, MT1-MMP, TIMP-1*, and *TIMP-2* mRNA expressions. HT-1080 cells were incubated with capsaicin and/or EGF (100 ng/mL) for 24 h. *MMP-9, MMP-2, MT1-MMP, TIMP-1*, and *TIMP-2* mRNA expressions were analyzed by semi-quantitative RT-PCR or real time-PCR. *GAPDH* expression was included as an internal control. (E and F) Effects of capsaicin on *MMP-9* and *MMP-2* promoter activities. Cells were transfected with a WT-*MMP-9* or WT-*MMP-2* promoter-containing reporter vector, and then incubated with various concentrations of capsaicin in the absence or presence of EGF (100 ng/mL), as indicated. Luciferase activity was measured 24 h after transfection. *Significantly different from EGF treatment only ($p<0.01$).

that the *MMP-9* promoter contains capsaicin-responsive elements. Capsaicin also affected the *luciferase* reporter gene containing the *MMP-2* promoter (Fig. 2F). No cytotoxicity was observed in the cells exposed to capsaicin (data not shown).

3.3 Capsaicin suppresses EGF-induced MT1-MMP expression

MT1-MMP has been shown to be a key enzyme in tumor metastasis. Therefore, we determined whether capsaicin suppressed its expression in HT-1080 cells stimulated with EGF. Western-blot and semi-quantitative RT-PCR analyses revealed that capsaicin suppressed EGF-induced *MT1-MMP* mRNA (Fig. 2B) and protein expression (Fig. 2A). As MMP activity is tightly regulated by endogenous inhibitors, the TIMPs [33], we further examined the expression level of TIMP-1 and TIMP-2 by semi-quantitative RT-PCR, but their expression level was essentially unchanged by treatment with capsaicin (Fig. 2B).

3.4 Capsaicin inhibits the transcriptional activity of MMP-9 through the suppression of EGF-stimulated AP-1 activity

The expression of *MMP-9* is regulated through the transcriptional-level interaction of AP-1 and NF-κB with their binding sequences in the *MMP-9* promoter [7, 28]. To test which of these transcription factors regulates *MMP-9* in

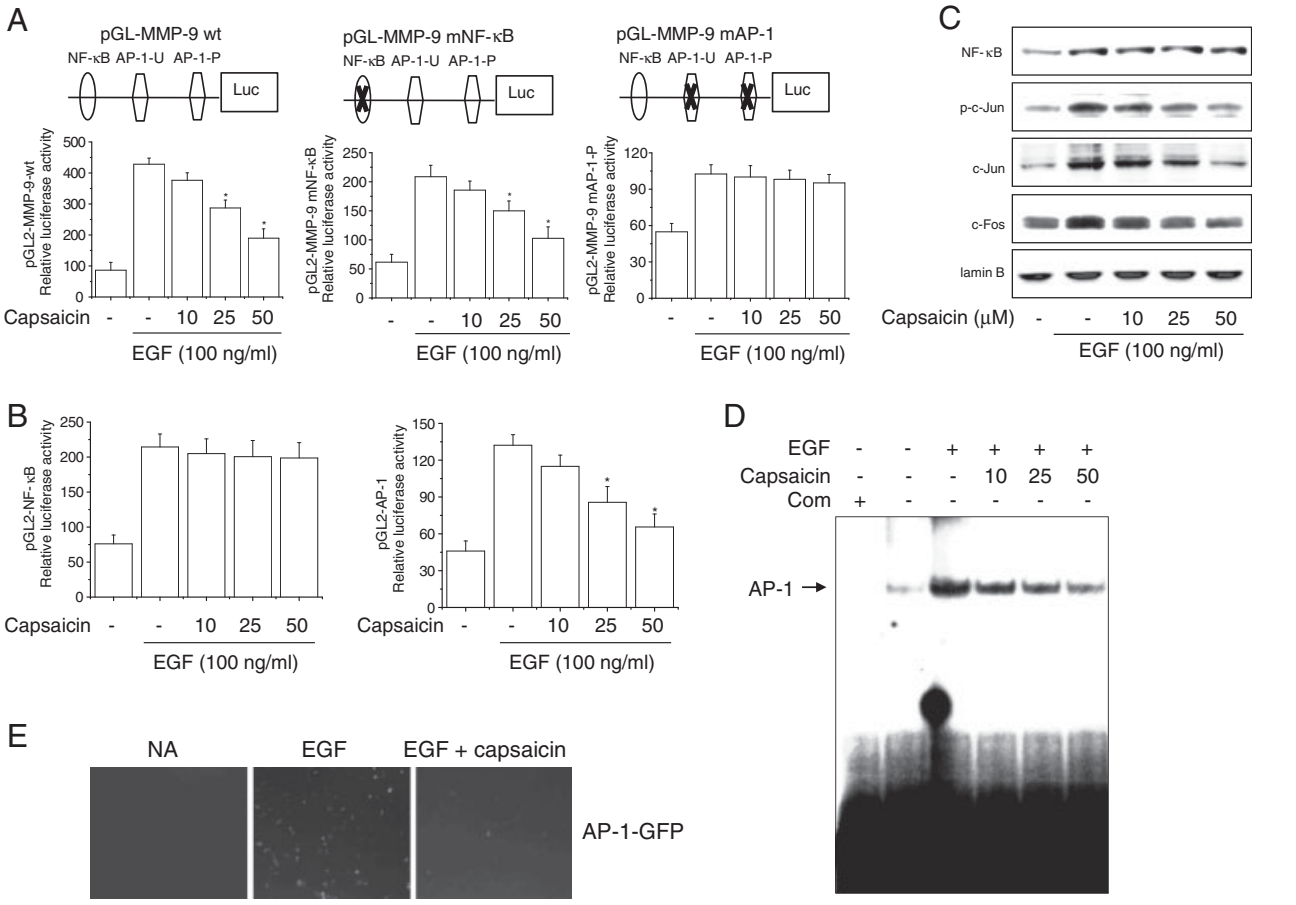

Figure 3. Inhibitory effects of capsaicin on the EGF-induced activation of AP-1. Mutations were introduced in the NF-κB or AP-1 binding sites of pGL-MMP-9WT. HT-1080 cells were transfected with pGL-MMP-9WT, pGL-MMP-9mNF-κB, or pGL-MMP-9mAP reporter plasmids (A) or with reporter plasmids containing tandem NF-κB or AP-1 binding sites (B). Cells were cultured with capsaicin and/or EGF for 24 h, and the relative luciferase activity in cell extracts was determined. *Significantly different from EGF treatment only ($p<0.01$). (C) Effects of capsaicin on EGF-induced c-Jun, c-Fos, and NF-κB nuclear translocations. HT-1080 cells were pretreated with capsaicin (10, 25, and 50 μM) for 1 h, and then treated with EGF (100 ng/mL) for 6 h. The nuclear extracts were subjected to SDS-PAGE, followed by Western blotting with antibodies against c-Jun, c-Fos, phospho-c-Jun, NF-κB, and lamin B. (D) Effect of capsaicin on the EGF-induced DNA-binding activity of AP-1. Cells were treated with EGF (100 ng/mL) and/or capsaicin (10, 25, and 50 μM) for 3 h. Nuclear extracts were then prepared for an EMSA. An autoradiograph of a dried gel, which is representative of three independent experiments, is shown. (E) Effects of capsaicin on EGF-induced AP-1 activation. Cells were transiently transfected with Lenti-AP-1-GFP. GFP fluorescence was analyzed by fluorescence microscopy.

HT-1080 cells, cells were transiently transfected with reporter genes that included the wild-type *MMP-9* promoter or a promoter with mutations in the NF-κB site or in one or both AP-1 sites (Fig. 3A). As shown in Fig. 3A, treatment with capsaicin in the presence of EGF did not affect the transcriptional activity of the reporter with the AP-1 mutations, suggesting that the targets of capsaicin were the AP-1 transcription factors. To further determine the promoter structure used by capsaicin, we transfected HT-1080 cells with pNF-κBLuc or pAP-1-Luc plasmid DNAs, which contained the *luciferase* gene driven by NF-κB- $(5'\text{-GGGGACTTTCC-}3')_n$ $(n = 5)$ or AP-1- $(5'\text{-TGACTAA-}3')_n$ $(n = 7)$ responsive elements, respectively. Transfected HT-1080 cells were treated with capsaicin for 24 h, and luciferase activity was determined. As shown in Fig. 3B, capsaicin suppressed the AP-1-containing promoters in a dose-dependent manner. HT-1080 cells were incubated with different concentrations of capsaicin in the presence of EGF for 6 h, and nuclear extracts were prepared and examined by Western-blot analysis. As shown in Fig. 3C, EGF induced the nuclear translocation of c-Jun and c-Fos, and capsaicin inhibited the nuclear translocation of c-Jun and c-Fos dose-dependently. However, capsaicin did not inhibit the EGF-induced nuclear translocation of NF-κB. Furthermore, capsaicin inhibited the phosphorylation of c-Jun in a dose-dependent manner.

To further investigate the inhibitory effect of capsaicin on the transcriptional activity of *MMP-9*, we examined the effect of capsaicin on the DNA-binding activity of AP-1 using an EMSA. When HT-1080 cells were treated with EGF for 3 h after pretreatment with capsaicin for 1 h, EGF-induced increases in the DNA-binding activity of AP-1 were substantially inhibited by capsaicin (Fig. 3D). To confirm the specificity of the capsaicin-mediated inhibitory effects on AP-1 in HT-1080 cells, cells were transiently transfected with reporter vectors that included tandem repeats of the AP-1 binding sites. As shown in Fig. 3E, treatment with capsaicin decreased EGF-mediated GFP fluorescence. No cytotoxicity was observed in the cells exposed to capsaicin (data not shown). Collectively, these data suggest that capsaicin regulates the transcriptional activation of *MMP-9* through the inhibition of EGF-stimulated AP-1, but not NF-κB, activity.

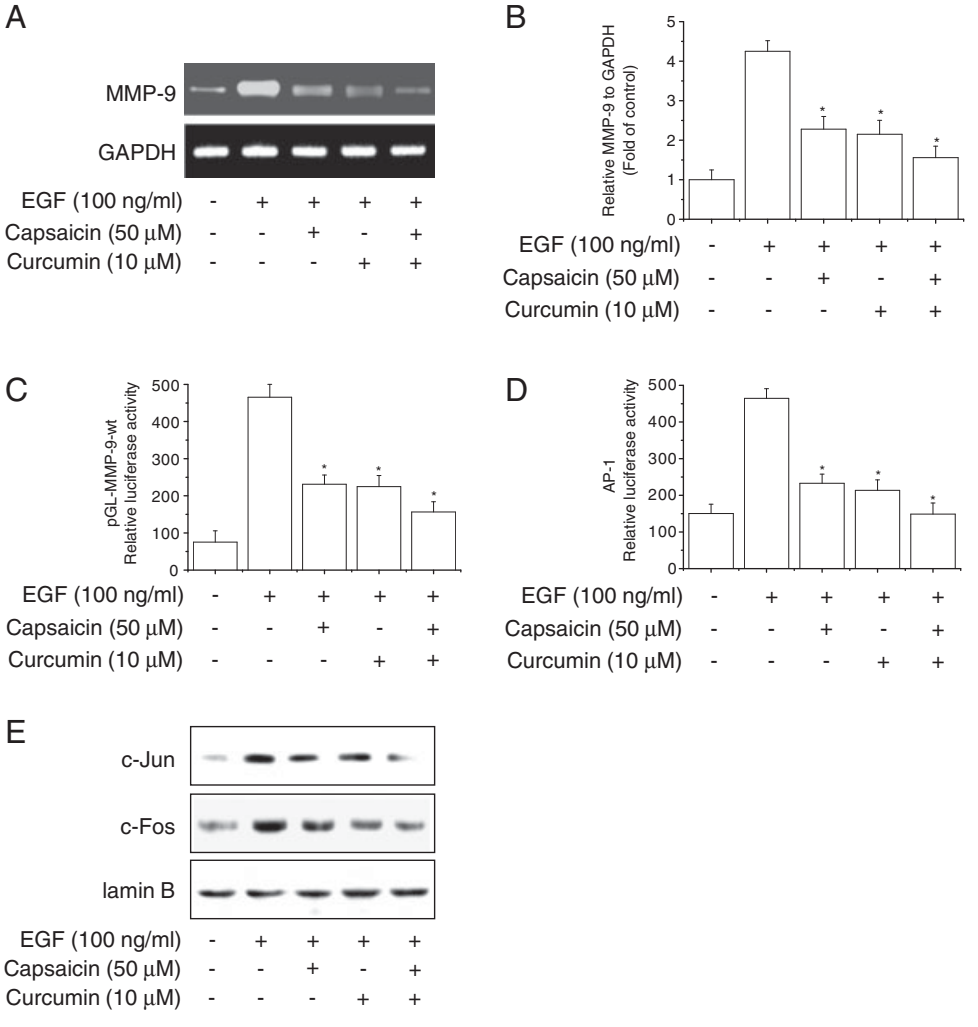

Figure 4. AP-1 is involved in the capsaicin-mediated down-regulation of *MMP-9*. Cells were treated with EGF (100 ng/mL) for 24 h in the absence or presence of capsaicin (50 μM) or curcumin (10 μM). (A) Conditioned media was collected after 24 h and gelatin zymography was performed. (B) *MMP-9* mRNA expression was analyzed by real time-PCR. *GAPDH* expression was included as an internal control. *Significantly different from EGF treatment only ($p < 0.01$). (C and D) Cells were transfected with pGL-MMP-9WT or pAP-1 reporter plasmids, and then cultured with capsaicin or curcumin and/or EGF for 24 h. Luciferase activity in cell extracts was determined. *Significantly different from EGF treatment only ($p < 0.01$). (E) Cells were pretreated with capsaicin or curcumin for 1 h, and then treated with EGF (100 ng/mL) for 6 h. The nuclear extracts were subjected to SDS-PAGE, followed by Western blotting with antibodies against c-Jun, c-Fos, and lamin B.

3.5 Capsaicin inhibits MMP-9 activation by suppressing EGF-stimulated AP-1 activity

We demonstrated that capsaicin inhibited the EGF-induced invasion of HT-1080 cells by suppressing MMP-9 expression through the regulation of AP-1 activity. Curcumin is reported to inhibit the activation of AP-1 induced by tumor promoters by direct interaction with the AP-1 DNA-binding motif and to inhibit JNK activation by carcinogens [34, 35]. Next, we examined whether the activation of AP-1 was involved in EGF-induced MMP-9 expression in HT-1080 cells using an AP-1 inhibitor, curcumin [36]. HT-1080 cells were pretreated with curcumin (10 μM) for 1 h and then stimulated with EGF (100 ng/mL) in the presence or absence of capsaicin (50 μM) for 24 h. Our results indicate that curcumin inhibited EGF-induced *MMP-9* expression, and combined treatment with curcumin and capsaicin additively reduced EGF-induced *MMP-9* expression (Fig. 4A and B). To test which of these transcription factors regulates *MMP-9* in HT-1080 cells, cells were transiently transfected with reporter genes that included the wild-type *MMP-9* promoter or pAP-1-Luc plasmid DNA, which contained the *luciferase* gene driven by AP-1-responsive elements. As shown in Fig. 4C and D, curcumin inhibited the EGF-induced transcriptional activation of *MMP-9* and AP-1 activities, and combined treatment with capsaicin additively reduced the EGF-induced transcriptional activation of *MMP-9* and AP-1 activities. Furthermore, as shown in Fig. 4E, curcumin or combined treatment with capsaicin inhibited the EGF-induced nuclear translocation of c-Jun and c-Fos.

3.6 Capsaicin suppresses the EGF-mediated phosphorylation of EGFR, FAK, Akt, PKC, Raf, ERK, and p38 MAPK

EGF binding to EGFR results in receptor dimerization, autophosphorylation, and the activation of various downstream signaling molecules, including FAK, PKC, PI3K/Akt, Ras, and MAPK. As expected, Western-blot analysis revealed that the treatment of HT-1080 cells with EGF led to the increased phosphorylation of EGFR, FAK, PKC, Akt, Raf, and MAPKs. Capsaicin inhibited the EGF-induced phosphorylation of EGFR, FAK, PKC, Akt, Raf, ERK1/2, and p38 MAPK, but had no effect on JNK1/2 phosphorylation (Fig. 5).

3.7 Capsaicin suppresses the invasion and migration of HT-1080 cells by inhibiting EGFR-mediated FAK/Akt, PKC/Raf/ERK, p38 MAPK, and AP-1 signaling

Subsequent experiments were designed to determine which of these signal transduction pathways are involved in EGF-stimulated MMP-9 expression and the inhibition of MMP-9

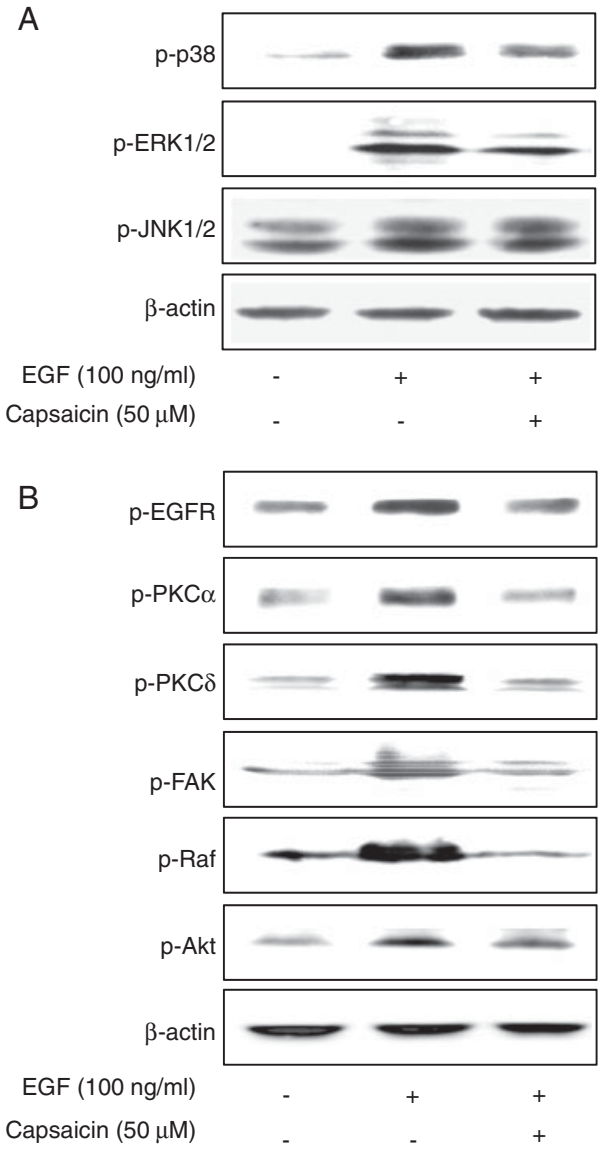

Figure 5. Capsaicin inhibits the EGF-induced phosphorylation of EGFR, FAK, Akt, PKC, Raf, ERK, and p38 MAPK in HT-1080 cells. Cells were treated with EGF (100 ng/mL) for 30 min in the presence or absence of capsaicin, and the phosphorylation of EGFR, FAK, Akt, PKC, Raf (B), and p38 MAPK, ERK 1/2, JNK 1/2 (A) was measured by Western blotting. β-actin was included as an internal control.

expression by capsaicin in HT-1080 cells. First, the effects of specific kinase inhibitors on MMP-9 expression in EGF-induced HT-1080 cells were analyzed by gelatin zymography and real-time-PCR. In addition, the effects of specific kinase inhibitors on AP-1 activity in EGF-induced HT-1080 cells were analyzed using luciferase assays. EGF-induced MMP-9 secretion (Fig. 6A) and the mRNA expression of *MMP-9* (Fig. 6B) were completely inhibited by inhibitors of EGFR (AG 1478), PKC (Gö 6976 or Rottlerin), PI3K/Akt (LY 294002), ERK (PD 98059), and p38 MAPK (SB 203580).

Next, we investigated whether capsaicin inhibited the activation of these signaling pathways. Since capsaicin inhibited EGFR phosphorylation and AP-1 activity, we examined whether the EGFR inhibitor AG 1478 inhibited MMP-9 activation (Fig. 6C). Treatment with 10 μM AG 1478 led to a significant decrease in the phosphorylation of FAK, PKC, Akt, Raf, ERK, and p38 MAPK (Fig. 6D). In addition, AG 1478 inhibited the EGF-induced nuclear translocation of c-Jun and c-Fos (Fig. 6E). Furthermore, the EGFR inhibitor significantly inhibited EGF-induced AP-1 activity (Fig. 6F). In addition, as shown in Fig. 7A and B, wound-healing and matrigel invasion assays indicated that the migration and invasion of HT-1080 cells was inhibited by an EGFR inhibitor.

4 Results and discussion

Natural compounds with anti-carcinogenic activities interfere with the initiation, development, and progression of cancer through the modulation of various mechanisms, including cellular proliferation, differentiation, apoptosis, angiogenesis, and metastasis. Capsaicin is an active component of red chili peppers of the genus *Capsicum* [21]. A recent report revealed that capsaicin had strong apoptotic activity in B16-F10 melanoma cells *via* the down-regulation of Bcl-2 [37]. Furthermore, Shin *et al.* [27] reported that capsaicin inhibited the migration of B16-F10 cells via the PI3K/Akt/Rac1 signaling pathway. Recently, many studies have demonstrated that capsaicin exerts antitumor activity in cancer cells. However, the effect of capsaicin against EGF-induced MMP-9 expression and the invasiveness of HT-1080 fibrosarcoma cells remain unclear. Here, we demonstrated that capsaicin suppressed cell invasion through the inhibition of MMP-9 expression with its detailed molecular mechanisms, supporting previous reports of its therapeutic potential in cancer.

In the present study, we found that the EGF-induced migration and invasion of HT-1080 cells (Fig. 1) and the EGF-stimulated secretion of MMP-9 and MMP-2 (Fig. 2A) were suppressed by capsaicin, suggesting that capsaicin has

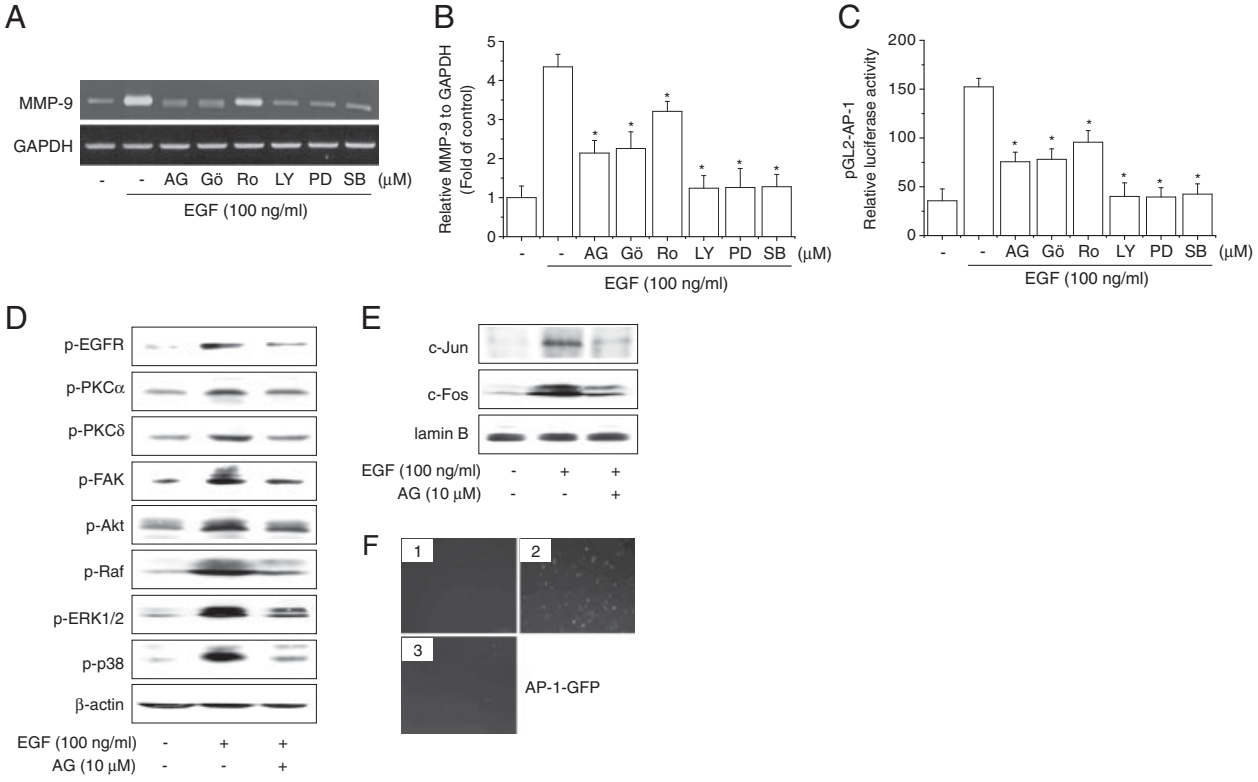

Figure 6. Capsaicin inhibits EGF-induced MMP-9 expression through the inhibition of EGFR-mediated FAK/Akt, PKC/Raf/ERK, and p38 MAPK in HT-1080 cells. (A–C) Effects of specific inhibitors on EGF-induced MMP-9 expression and AP-1 activation. Cells were pretreated for 1 h with AG 1478 (AG, 10 μM), Gö 6976 (Gö, 2 μM), Rottlerin (RO, 1 μ), LY 294002 (LY, 10 μM), PD 98059 (PD, 30 μM) or SB 203580 (SB, 20 μM) followed by EGF (100 ng/mL) stimulation for 24 h. MMP-9 activity in the conditioned media was analyzed by gelatin zymography (A). *MMP-9* mRNA expression was analyzed by real time-PCR. *Significantly different from EGF treatment only ($p<0.01$) (B). The relative luciferase activity in the cell extracts was determined (C). *Significantly different from EGF treatment only ($p<0.01$). (D) Cells were stimulated with EGF for 30 min after pretreatment with AG 1478 (10 μM) for 1 h, and the phosphorylation levels of EGFR, PKCα, PKCδ, FAK, Raf, Akt, ERK, and p38 MAPK were determined. (E) Cells were pretreated with AG 1478 (10 μM) for 1 h followed by EGF stimulation for 6 h. Western blotting was performed to determine the nuclear levels of c-Jun and c-Fos. (F) Cells were transiently transfected with Lenti-AP-1-GFP. GFP fluorescence was analyzed by fluorescence microscopy.

potential as an inhibitor of fibroblast cancer progression. In EGF-treated HT-1080 cells, capsaicin also suppressed the increased expression of MMP-9 and MMP-2 (Fig. 2B–F). Neither the protein levels of TIMP-1 nor TIMP-2 were altered by capsaicin in our system, possibly because of the different systems and concentrations. The role of MT1-MMP in pericellular proteolysis demonstrated that it may also directly influence ECM turnover [38]. Moreover, it was identified as the first physiological activator of pro-MMP-2 [5]. Therefore, the inhibition of ECM-degrading enzymes and cell adhesion molecules could be a preventive approach for cancer metastasis. In this study, we found that capsaicin significantly reduced MT1-MMP (Fig. 2A and B).

We further investigated the mechanism of *MMP-9* transcriptional regulation by capsaicin. The *MMP-9* promoter contains *cis*-acting regulatory elements for transcription factors, including two AP-1 sites and an NF-κB site [7, 28]. Capsaicin suppressed *MMP-9* induction by repressing the transcriptional activation of the *MMP-9* promoter (Fig. 3A). Mutational analysis of the promoter revealed that the major target of capsaicin was the AP-1 site, a finding that was further confirmed by the use of reporter plasmids containing synthetic elements specific for these transcription factors (Fig. 3B). We found that capsaicin inhibited the translocation of c-Jun and c-Fos, both of which are members of the AP-1 family, to the nucleus in EGF-treated HT-1080 cells. These results clearly indicate that capsaicin inhibited *MMP-9* activation by reducing the transcription factor AP-1. Therefore, AP-1 is an important transcription factor for *MMP-9* expression, which in turn promotes cancer cell invasion and migration, and capsaicin is likely a potent universal inhibitor for AP-1 activation. Capsaicin inhibited the phosphorylation of c-Jun in whole-cell lysates (Fig. 3C). It also blocked the translocation of c-Jun to the nucleus in EGF-treated HT-1080 cells. However, capsaicin did not suppress the EGF-induced nuclear translocation of NF-κB (Fig. 3C). We then confirmed AP-1 transcriptional activity in HT-1080 cells by transiently expressing the AP-1 reporter. As shown in Fig. 3E, capsaicin decreased EGF-mediated AP-1-GFP fluorescence. In addition, EGF-induced increases in the DNA-binding activity of AP-1 were substantially inhibited by capsaicin (Fig. 3D).

AP-1 controls the transcription of many genes, including *MMP-9*. Therefore, we investigated the functional significance of AP-1 transactivation in *MMP-9* activation in HT-1080 cells. Treatment with curcumin [36, 39], a potent inhibitor of AP-1 transcriptional activation, reduced EGF-induced enzymatic activity and MMP-9 expression (Fig. 4A–C). The inhibitor also reduced the EGF-induced transcriptional activity of AP-1 and nuclear translocation of c-Jun and c-Fos (Fig. 4D and E). Furthermore, combined treatment with curcumin and capsaicin additively reduced EGF-induced *MMP-9* expression and the nuclear translocation of c-Jun and c-Fos (Fig. 4). These findings collectively suggest that capsaicin inhibited the EGF-induced activation of *MMP-9* by suppressing AP-1 activation in fibroblasts.

The activation of EGFR tyrosine kinase by EGF binding leads to cell proliferation and cancer cell metastasis via MMP-9 expression. Important signaling molecules, such as PKCs, FAK, Ras-MAPK, STAT-3, and PI-3 kinase, are associated with the EGF/EGFR pathway, which also regulates many processes associated with tumor metastasis [13, 14]. In this study, we identified the signaling pathway-mediated regulation of *MMP-9* in EGF-induced HT-1080 cells in response to capsaicin treatment. Capsaicin suppressed the EGF-induced phosphorylation of EGFR, FAK, PKC, Akt, Raf, ERK1/2, and p38 MAPK (Fig. 5). To clarify the EGF-induced signaling cascade underlying MMP-9 expression in human HT-1080 fibrosarcoma cells, we assessed the effects of specific inhibitors of EGFR (AG 1478), PKC (Gö 6976 or Rottlerin), PI3K/Akt (LY 294002), ERK (PD 98059), and p38 MAPK (SB 203580) on EGF-induced MMP-9 expression and transcriptional activity. EGF-induced MMP-9 expression and transcriptional activity were significantly inhibited by treatment with the EGFR, PKC, PI3K/Akt, ERK, and p38 MAPK inhibitors

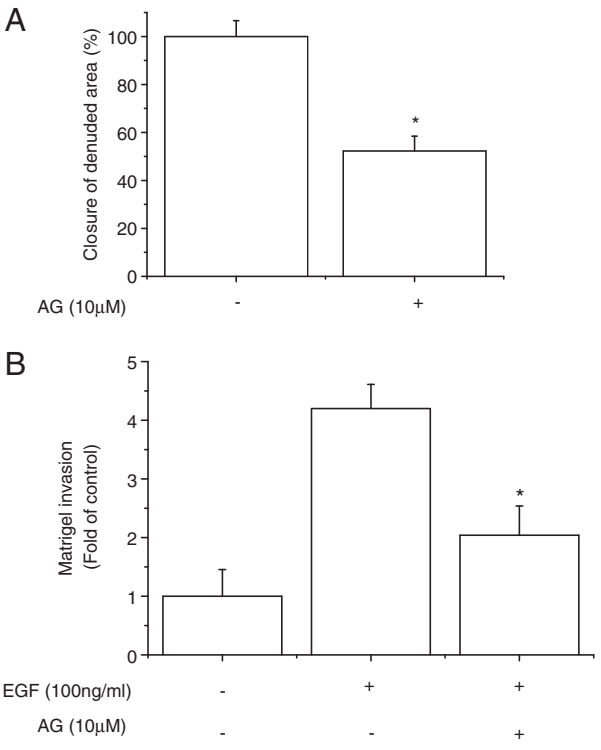

Figure 7. Effects of AG1478 on the migration and invasion of HT-1080 cells. (A) Cells were scratched with a pipette tip and then treated with AG 1478 (10 μM) for 24 h. Migrating cells were imaged by phase-contrast microscopy. The data are expressed as the mean ± SD of triplicate experiments. *Significantly different from the control ($p < 0.01$). (B) Cells were pre-treated with AG 1478 (10 μM) followed by EGF treatment (100 ng/mL) for 24 h. The cells on the bottom of the filter were then counted. The data are expressed as the mean ± SD of triplicate experiments. *Significantly different from EGF treatment only ($p < 0.01$).

(Fig. 6A–C). In addition, AG 1478 caused a significant decrease in the phosphorylation of FAK, PKC, Akt, Raf, MEK/ERK, and p38 MAPK (Fig. 6D). In addition, AG 1478 inhibited the EGF-induced nuclear translocation of c-Jun and c-Fos (Fig. 6E). Furthermore, the EGFR inhibitor significantly inhibited EGF-induced AP-1 activity (Fig. 6C and F), as well as EGF-induced MMP-9 expression (Fig. 6A and B), cell migration (Fig. 7A), and invasion (Fig. 7B). These results indicate that EGF-induced *MMP-9* expression is primarily regulated by EGFR, PKC, PI3K/Akt, ERK, and p38 MAPK.

Accumulating evidence suggests that capsaicin has a diverse range of molecular targets, supporting the concept that it acts upon numerous biochemical and molecular cascades. This polyphenol modulates various targets either through direct interaction or through modulation of gene expression. Various molecular targets modulated by this agent include transcription factors, growth factors and their receptors, cytokines, enzymes, and genes regulating cell proliferation and apoptosis [40]. In summary, the present data demonstrate that capsaicin inhibits the EGF-induced invasion and migration of human fibrosarcoma cells via EGFR-mediated FAK/Akt, PKC/Raf/ERK, p38 MAPK, and AP-1 cascades, with consequent suppression of MMP-9 expression. Capsaicin, therefore, has potential as a potent anti-cancer drug in the treatment of fibrosarcoma metastasis.

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5 References

[1] Liotta, L. A., Steeg, P. S., Stetler-Stevenson, W. G., Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell* 1991, *64*, 327–336.

[2] Deryugina, E. I., Luo, G. X., Reisfeld, R. A., Bourdon, M. A. *et al.*, Tumor cell invasion through matrigel is regulated by activated matrix metalloproteinase-2. *Anticancer Res.* 1997, *17*, 3201–3210.

[3] Westermarck, J., Kahari, V. M., Regulation of matrix metalloproteinase expression in tumor invasion. *FASEB J.* 1999, *13*, 781–792.

[4] Van den Steen, P., Dubois, B., Nelissen, I., Rudd, P. *et al*, Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). *Crit. Rev. Biochem. Mol. Biol.* 2002, *37*, 375–536.

[5] Sato, H., Takino, T., Okada, Y., Cao, J. *et al.*, A matrix metalloproteinase expressed on the surface of invasive tumour cells. *Nature* 1994, *370*, 61–65.

[6] Yan, C., Boyd, D. D., Regulation of matrix metalloproteinase gene expression. *J. Cell. Physiol.* 2007, *211*, 19–26.

[7] Cho, H. J., Kang, J. H., Kwak, J. Y., Lee, T. S. *et al.*, Ascofuranone suppresses PMA-mediated matrix metalloproteinase-9 gene activation through the Ras/Raf/MEK/ERK- and Ap1-dependent mechanisms. *Carcinogenesis* 2007, *28*, 1104–1110.

[8] Kajanne, R., Miettinen, P., Mehlem, A., Leivonen, S. K. *et al.*, EGF-R regulates MMP function in fibroblasts through MAPK and AP-1 pathways. *J. Cell. Physiol.* 2007, *212*, 489–497.

[9] Liu, Z., Klominek, J., Regulation of matrix metalloproteaseactivity in malignant mesothelioma cell lines by growth factors. *Thorax* 2003, *58*, 198–203.

[10] Nutt, J., Durkan, G., Mellon, J., Lunec, J., Matrix metalloproteinases (MMPs) in bladder cancer: the induction of MMP9 by epidermal growth factor and its detection in urine. *BJU Int.* 2003, *91*, 99–104.

[11] Marmor, M. D., Skaria, K. B., Yarden, Y., Signal transduction and oncogenesis by ErbB/HER receptors. *Int. J. Radiat. Oncol. Biol. Phys.* 2004, *58*, 903–913.

[12] Yatabe, Y., Takahashi, T., Mitsudomi, T., Epidermal growth factor receptor gene amplification is acquired in association with tumor progression of EGFR-mutated lung cancer. *Cancer Res.* 2008, *68*, 2106–2111.

[13] Prenzel, N., Fischer, O. M., Streit, S., Hart, S. *et al.*, The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. *Endocr. Relat. Cancer* 2001, *8*, 11–31.

[14] Yarden, Y., Sliwkowski, M. X., Untangling the ErbB signalling network. *Nat. Rev. Mol. Cell Biol.* 2001, *2*, 127–137.

[15] Cheresh, D. A., Leng, J., Klemke, R. L., Regulation of cell contraction and membrane ruffling by distinct signals in migratory cells. *J. Cell Biol.* 1999, *146*, 1107–1116.

[16] Glading, A., Chang, P., Lauffenburger, D. A., Wells, A., Epidermal growth factor receptor activation of calpain is required for fibroblast motility and occurs via an ERK/MAP kinase signaling pathway. *J. Biol. Chem.* 2000, *275*, 2390–2398.

[17] Carlomagno, F., Santoro, M., Receptor tyrosine kinases as targets for anticancer therapeutics. *Curr. Med. Chem.* 2005, *12*, 1773–1781.

[18] Surh, Y. J., Cancer chemoprevention with dietary phytochemicals. *Nat. Rev. Cancer* 2003, *3*, 768–780.

[19] Sporn, M. B., Suh, N., Chemoprevention of cancer. *Carcinogenesis* 2000, *21*, 525–530.

[20] Lippman, S. M., Hong, W. K., Cancer prevention science and practice. *Cancer Res.* 2002, *62*, 5119–5125.

[21] Cordell, G. A., Araujo, O. E., Capsaicin: Identification, nomenclature, and pharmacotherapy. *Ann. Pharmacother.* 1993, *27*, 330–336.

[22] Matucci Cerinic, M., McCarthy, G., Lombardi, A., Pignone, A. *et al.*, Neurogenic influences in arthritis: potential modification by capsaicin. *J. Rheumatol.* 1995, *22*, 1447–1449.

[23] Sindrup, S. H., Jensen, T. S., Efficacy of pharmacological treatments of neuropathic pain: an update and effect related to mechanism of drug action. *Pain* 1999, *83*, 389–400.

[24] Aggarwal, B. B., Shishodia, S., Molecular targets of dietary agents for prevention and therapy of cancer. *Biochem. Pharmacol.* 2006, *71*, 1397–1421.

[25] Amantini, C., Mosca, M., Nabissi, M., Lucciarini, R. *et al.*, Capsaicin-induced apoptosis of glioma cells is mediated by TRPV1 vanilloid receptor and requires p38 MAPK activation. *J. Neurochem.* 2007, *102*. 977–990.

[26] Huang, S. P., Chen, J. C., Wu, C. C., Chen, C. T. *et al.*, Capsaicin-induced apoptosis in human hepatoma HepG2 cells. *Anticancer Res.* 2009, *29*, 165–174.

[27] Shin, D. H., Kim, O. H., Jun, H. S., Kang, M. K., Inhibitory effect of capsaicin on B16-F10 melanoma cell migration via the phosphatidylinositol 3-kinase/Akt/Rac1 signal pathway. *Exp. Mol. Med.* 2008, *40*, 486–494.

[28] Eberhardt, W., Schulze, M., Engels, C., Klasmeier, E. *et al.*, Glucocorticoid-mediated suppression of cytokine-induced matrix metalloproteinase-9 expression in rat mesangial cells: involvement of nuclear factor-kappaB and Ets transcription factors. *Mol. Endocrinol.* 2002, *16*, 1752–1766.

[29] Lee, K. J., Hwang, S. J., Choi, J. H., Jeong, H. G., Saponins derived from the roots of Platycodon grandiflorum inhibit HT-1080 cell invasion and MMPs activities: regulation of NF-kappaB activation via ROS signal pathway. *Cancer Lett.* 2008, *268*, 233–243.

[30] Johnson, M. R., Wang, K., Smith, J. B., Heslin, M. J. *et al.*, Quantitation of dihydropyrimidine dehydrogenase expression by real-time reverse transcription polymerase chain reaction. *Anal. Biochem.* 2000, *278*, 175–184.

[31] Hwang, Y. P., Yun, H. J., Kim, H. G., Han, E. H. *et al.*, Suppression of PMA-induced tumor cell invasion by dihydroartemisinin via inhibition of PKCalpha/Raf/MAPKs and NF-kappaB/AP-1-dependent mechanisms. *Biochem. Pharmacol.* 2010, *79*, 1714–1726.

[32] Han, E. H., Kim, J. Y., Kim, H. K., Hwang, Y. P. *et al.*, o,p'-DDT induces cyclooxygenase-2 gene expression in murine macrophages: Role of AP-1 and CRE promoter elements and PI3-kinase/Akt/MAPK signaling pathways. *Toxicol. Appl. Pharmacol.* 2008, *233*, 333–342.

[33] Kazes, I., Elalamy, I., Sraer, J. D., Hatmi, M. *et al*, Platelet release of trimolecular complex components MT1-MMP/TIMP2/MMP2: involvement in MMP2 activation and platelet aggregation. *Blood* 2000, *96*, 3064–3069.

[34] Dickinson, D. A., Iles, K. E., Zhang, H., Blank, V. *et al.*, Curcumin alters EpRE and AP-1 binding complexes and elevates glutamate-cysteine ligase gene expression. *FASEB J.* 2003, *17*,473–475.

[35] Hahm, E. R., Gho, Y. S., Park, S., Park, C. *et al.*, Synthetic curcumin analogs inhibit activator protein-1 transcription and tumor-induced angiogenesis. *Biochem. Biophys. Res. Commun.*2004, *321*, 337–344.

[36] Wang, H. H., Hsieh, H. L., Wu, C. Y., Sun, C. C. *et al.*, Oxidized low-density lipoprotein induces matrix metalloproteinase-9 expression via a p42/p44 and JNK-dependent AP-1 pathway in brain astrocytes. *Glia* 2009, *57*, 24–38.

[37] Jun, H. S., Park, T., Lee, C. K., Kang, M. K. *et al.*, Capsaicin induced apoptosis of B16-F10 melanoma cells through down-regulation of Bcl-2. *Food Chem. Toxicol.* 2007, *45*, 708–715.

[38] Egeblad, M., Werb, Z., New functions for the matrix metalloproteinases in cancer progression. *Nat. Rev. Cancer* 2002, *2*, 161–174.

[39] Surh, Y. J., Han, S. S., Keum, Y. S., Seo, H. J. *et al.*, Inhibitory effects of curcumin and capsaicin on phorbol ester-induced activation of eukaryotic transcription factors, NF-kappaB and AP-1. *Biofactors* 2000, *12*, 107–112.

[40] Oyagbemi, A. A., Saba, A. B., Azeez, O. I., Capsaicin: a novel chemopreventive molecule and its underlying molecular mechanisms of action. *Indian J. Cancer* 2010, *47*, 53–58.

