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RESEARCH PAPER

Metformin blocks migration and invasion of tumour cells by inhibition of matrix metalloproteinase-9 activation through a calcium and protein kinase Cα-dependent pathway: phorbol-12-myristate-13-acetate-induced/extracellular signal-regulated kinase/activator protein-1

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Background and purpose: Population studies have revealed that treatment with the anti-diabetic drug metformin is significantly associated with reduced cancer risk, but the underlying mode of action has not been elucidated. The aim of our study was to determine the effect of metformin on tumour invasion and migration, and the possible mechanisms, using human fibrosarcoma HT-1080 cells.
Experimental approach: We employed invasion, migration and gelatin zymography assays to characterize the effect of metformin on HT-1080 cells. Transient transfection assays were performed to gene promoter activities, and immunoblot analysis to study its molecular mechanisms of action.
Key results: Metformin inhibited migration and invasion by HT-1080 cells at sub-toxic concentrations. In these cells, metformin also suppressed phorbol-12-myristate-13-acetate (PMA)-enhanced levels of matrix metalloproteinases-9 (MMP-9) protein, mRNA and transcription activity through suppression of activator protein-1 (AP-1) activation. In addition, metformin strongly repressed the PMA-induced phosphorylation of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and protein kinase C(PKC)α, whereas the phosphorylation of p38 mitogen-activated protein kinase was not affected by metformin. Metformin decreased the PMA-induced Ca^{2+} influx. Furthermore, treatment with an intracellular Ca^{2+} chelator (BAPTA-AM) or a selective calmodulin antagonist (W7) markedly decreased PMA-induced MMP-9 secretion and cell migration, as well as activation of ERK and JNK/AP-1.
Conclusions and implications: Metformin inhibited PMA-induced invasion and migration of human fibrosarcoma cells via Ca^{2+}-dependent PKCα/ERK and JNK/AP-1-signalling pathways. Metformin therefore has the potential to be a potent anti-cancer drug in therapeutic strategies for fibrosarcoma metastasis.
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Keywords: metformin; MMP-9; migration; invasion; AP-1; PKCα; calcium signalling; anti-cancer

Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; AP-1, activator protein-1; CaM, calmodulin; CaMK, calmodulin-dependent kinase; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MMP-9, matrix metalloproteinases-9; NF-κB, nuclear factor-κB; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate

Introduction

Metformin is a commonly prescribed oral anti-hyperglycemic drug used in the management of type 2 diabetes. Recent evidence indicates that metformin has significant effects on tumourigenesis and cancer cell growth, as well as antioxidant

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effects. It has been reported that patients with type 2 diabetes who take metformin have a lower risk of cancer than similar patients who do not take metformin (Evans *et al*., 2005; Bowker *et al*., 2006). In a mouse xenograft model, metformin suppressed tumour growth of p53-negative HCT116 colon cancer cells, but not of p53 wild-type cells (Buzzai *et al*., 2007). Metformin treatment decreased the incidence and size of mammary adenocarcinomas in Her2/Neu mice (Anisimov *et al*., 2005), and prevented carcinogen-induced pancreatic cancer in hamsters (Schneider *et al*., 2001). In culture, metformin has been shown to inhibit growth of cells derived from breast cancer, colon cancer, prostate cancer and gliomas (Isakovic *et al*., 2007; Ben Sahra *et al*., 2008; Hirsch *et al*., 2009; Phoenix *et al*., 2009). In addition, a recent study revealed the ability of metformin to inhibit *in vitro* migration of malignant glioma cells (Beckner *et al*., 2005), indicating its potential usefulness in anti-cancer therapy. However, the mechanisms of action by which metformin mediates these beneficial effects against cancer cell invasion are not well understood.

Tumour invasion and metastasis are a major cause of cancer-related deaths and involve several biological processes. Cell–extracellular matrix (ECM) interactions, disconnection of intercellular adhesion, degradation of ECM and invasion of lymph and blood vessels are critical steps for cancer invasion and metastasis (Liotta *et al*., 1991; Deryugina *et al*., 1997). A number of proteolytic enzymes participate in the degradation of environmental barriers, such as the ECM and basement membrane (Westermarck and Kahari, 1999). Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases, play an important role in the proteolysis of various ECM components and are involved in the metastasis and angiogenesis of cancer cells (Westermarck and Kahari, 1999). In particular, MMPs are crucial in the proteolysis of ECM proteins, collagen and fibronectin (Johnson *et al*., 1998). MMPs are synthesized as pre-pro-enzymes and secreted from cells as pro-enzymes. Among the human MMPs reported to date, MMP-2 and -9 are the key enzymes involved in degrading types I and IV collagen, and ECM (Salo *et al*., 1994; Johnson *et al*., 1998). MMPs are a family of zinc-dependent proteases that are divided into four subclasses based on their substrate specificity; the subclasses include collagenase, gelatinase, stromelysin and membrane-associated MMPs (Yan and Boyd, 2007). Tumour-secreted MMPs destroy ECM components in the tissues surrounding the tumour, and tumour cells subsequently invade through the basement membrane of blood vessels and facilitate the spread to distant organs, resulting in organ failure and patient mortality. Both MMP-2 and -9, which are abundantly expressed in various malignant tumours, contribute to cancer invasion and metastasis (Johnsen *et al*., 1998). Generally, MMP-2 is constitutive and over-expressed in highly metastatic tumours, whereas MMP-9 can be stimulated by tumour necrosis factor-α (an inflammatory cytokine), by epidermal growth factor, or by the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate, through activation of different intracellular signalling pathways (Cho *et al*., 2007; Kajanne *et al*., 2007). Mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase are the predominant cascades that participate in MMP-9 expression. In addition, transcriptional regulation by activating transcription factors including activator protein-1 (AP-1), nuclear factor-κB (NF-κB) or Sp-1 is also reported to occur in the regulation of MMP-9 gene expression. Furthermore, stimulators such as cytokines and phorbol-12-myristate-13-acetate (PMA) control the expression of MMP-9 by modulating the activation of transcription factors such as AP-1 and NF-κB through Ras/Raf/extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), phosphatidylinositol 3-kinase (PI3K)/AKT and protein kinase C (PKC) signalling pathways (Kim and Suh, 2001; Sato *et al*., 2002), because the promoter region of MMP-9 has AP-1 and NF-κB binding sites (Cho *et al*., 2007; Shin *et al*., 2007). Therefore, agents that can suppress the expression of MMP-2 or -9 should be developed as potential agents against cancer invasion and metastasis.

Recently, it has been suggested that calcium (Ca^{2+}) can regulate the expression or activation of MMPs. Ca^{2+} may be involved in controlling the activity of MMP-12 (Gossas and Danielson, 2006). Increased extracellular Ca^{2+} levels induce MMP-9 gene expression in human keratinocytes (Kobayashi *et al*., 2001; Mukhopadhyay *et al*., 2004), and the inhibition of Ca^{2+} influx decreases the level of MMP-1 mRNA (Kohn *et al*., 1994). Furthermore, modulation of intracellular Ca^{2+} levels can alter the secretion of MMP-1 from migrating keratinocytes (Sudbeck *et al*., 1997). Ca^{2+}/calmodulin (CaM) is a Ca^{2+}-binding protein that is implicated in a variety of cellular functions, including cell growth, proliferation and migration (Cheung, 2002; Mercure *et al*., 2008). Ca^{2+}/CaM itself does not show any catalytic activity, but regulates the activity of a number of Ca^{2+}/CaM-dependent enzymes, such as myosin light chain kinase (MLCK) (Walsh *et al*., 1979), Ca^{2+}/CaM-dependent kinase (CaMK) (Colbran and Soderling, 1990), phosphodiesterase (Rybalkin and Bornfeldt, 1999), protein phosphatase (Klee, 1991) and nitric oxide synthase (Schmidt *et al*., 1992). Previous studies have shown that tumour growth is inhibited by Ca^{2+} channel blockers (Catterall, 2000). Recent evidence has implicated Ca^{2+}/CaM in cancers, for example, abnormal expression of Ca^{2+}/CaM often occurs in certain tumours (Wei *et al*., 1982), and specific antagonists of Ca^{2+}/CaM inhibit the growth of a variety of tumour cells (Schuller *et al*., 1990; Strobl and Peterson, 1992; Shim *et al*., 2007). Thus, Ca^{2+}/CaM is a potential target for cancer chemotherapy (Hait and Lazo, 1986; Roderick and Cook, 2008). These reports indicate that inhibition of Ca^{2+} currents could be responsible for the anti-tumour activity of these drugs. Interestingly, a recent study revealed the ability of metformin to inhibit proliferation of vascular smooth muscle cells and to decrease agonist-induced intracellular Ca^{2+} responses (Sharma and Bhalla, 1995; Dominguez *et al*., 1996). In the present study, metformin significantly suppressed MMP-9 activation by blocking Ca^{2+} influx and the PKCα/ERK and JNK/AP-1-signalling pathways, which accounted for the reductions in migration and invasion of human fibrosarcoma cells. The present study shows for the first time that metformin is a Ca^{2+} antagonist, and provides a critical clue for delineating the molecular mechanisms underlying the inhibition of MMP-9 activation by metformin.

Methods

Cell culture and cell treatments
HT-1080 cells from the American Type Culture Collection (Manassas, VA, USA) were grown in RPMI1640 supplemented with 10% fetal bovine serum (FBS), 100 IU·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin at 37°C in a 5% CO_2 humidified incubator. Cells were treated with different concentrations of metformin in the absence or presence of PMA (30 nM) for 24 h. Metformin was dissolved in serum-free culture medium.

Measurement of cell viability
HT-1080 cells were plated at a density of 4×10^4 cells per 500 μL in 48-well plates, and the cell viability was determined by the conventional MTT reduction assay. After incubation, cells were treated with MTT solution (final concentration, 1 mg·mL^{-1}) for 1 h. The dark blue formazan crystals formed in intact cells were solubilized with DMSO, and the absorbance at 570 nm was measured with a microplate reader (Varioskan, Thermo Electron Co. Berthold, Germany).

In vitro *wound-healing assay*
HT-1080 cells were seeded in a six-well plate and grown overnight to confluence. The monolayer cells were scratched with a 200 μL pipette tip to create a wound, and washed twice with serum-free RPMI1640 to remove floating cells; the medium was then replaced with medium without serum. The rate of wound closure was assessed and photographed 24 h later. Each value is derived from three randomly selected fields.

Matrigel invasion assay
HT-1080 cells were incubated in RPMI1640 with 10% FBS, and then collected by trypsinization. Cells (1×10^5 cells·mL^{-1}) in serum-free medium were added to the inner cup of a 48-well Transwell chamber (Corning Life Sciences, Corning, New York, 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 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.

RNA preparation and semi-quantitative PCR
Total RNA was isolated with an RNA extraction kit (Amersham Pharmacia, Buckinghamshire, UK), and the concentration of total RNA was measured spectrophotometrically. RNA (2 μg) was converted to complementary DNA by an RT–PCR Bead kit (Amersham Pharmacia) 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. Amplified products were resolved by 1.5% agarose gel electrophoresis, stained with ethidium bromide and photographed under ultraviolet light.

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 of the reporter dye (SYBR). 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 (Johnson *et al*., 2000). The following primers were used in this study: MMP-2 forward: 5′- AGT CTG AAG AGC GTG AAG-3′, MMP-2 reverse: 5′- CCA GGT AGG AGT GAG AAT G-3′, MMP-9 forward: 5′- TGA CAG CGA CAA GAA GTG-3′, MMP-9 reverse: 5′- CAG TGA AGC GGT ACA TAG G-3′, GAPDH forward: 5′- CCA CCC ATG GCA AAT TCC-3′, GAPDH reverse: 5′- TGG GAT TTC CAT TGA TGA CAA -3′. The quantity of each transcript was calculated as described in the instrument manual and normalized to the amount of GAPDH, a housekeeping gene.

Western blotting analysis
After treatment, cells were collected and washed with phosphate-buffered saline. 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), 0.1% NP40) and centrifuged at 13 000× *g* for 15 min. Supernatants were collected and protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). Aliquots of the lysates (40 μg of protein) were boiled for 5 min and electrophoresed on a 10% SDS–polyacrylamide gel; the resolved proteins were then transferred to a PVDF membrane. The membrane was blocked with 1% BSA at room temperature for 1 h and then incubated with specific primary antibodies for 3 h, followed by incubation with the appropriate alkaline phosphatase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h. Finally, protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Pierce Biotechnology).

Gelatin zymography
The enzymatic activities of MMP-2 and MMP-9 were determined by gelatin zymography. Briefly, cells were seeded and allowed to grow to confluence for 24 h, and then maintained in serum-free medium. The conditioned media were collected 24 h after stimulation, mixed with non-reducing sample buffer and subjected to electrophoresis in a 10% polyacrylamide gel containing 0.1% (wt/vol) gelatin. The gel was washed with washing buffer containing 2.5% Triton X-100 and 50 mM Tris–HCl (pH 7.5), and incubated at 37°C for 24 h in 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM $CaCl_2$, 1 mM $ZnCl_2$ and 40 mmol·L^{-1} NaN_3. The gel was stained with 0.25% (wt/vol) Coomassie Brilliant Blue in 45% (vol/vol) methanol and 1% (vol/vol) acetic acid. Gelatinolytic activity was normalized against protein content of the cultured cells as assayed by the BCA kit.

Transient transfection and luciferase assay
To determine promoter activity, we used a dual luciferase reporter assay system (Promega, Madison, WI, USA). The cells were plated in 48-well plates and incubated at 37°C. At

Figure 1 Metformin suppression of the migration and invasion of human fibrosarcoma HT-1080 cells. (A) HT-1080 cells were incubated with varying concentrations of metformin in the presence of PMA (30 nM) or metformin only for 24 h in serum-free medium, and viability was determined by an MTT assay. Data are expressed as the means ± SD of triplicate experiments. *Significantly different from control ($P < 0.01$). (B) Cell monolayers were scratched with a pipette tip and then treated with metformin (1–5 mM) for 24 h. Migrating cells were photographed under phase contrast microscopy. Data are expressed as the means ± SD of triplicate experiments. *Significantly different from control ($P < 0.01$). (C) Cells were pretreated with metformin (5 mM) or vehicle (0.1% DMSO) followed by PMA (30 nM, 0.1% DMSO) treatment for 24 h. After 24 h, cells on the lower surface of the filter were counted. (1) Control; (2) PMA alone (30 nM); (3) PMA with Met (5 mM). Data are expressed as the means ± SD of triplicate experiments. *Significantly different from PMA treatment only ($P < 0.01$).

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-κB reporter vector (1 μg) and pCMV-β-gal (0.5 μg) plasmids were transfected using LipofectAMINE 2000 reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer's protocol. After incubation, the cells were lysed, and luciferase activity was measured using a luminometer (Luminoscan Ascent, Thermo Electron Co., Berthold, Germany). Luciferase activity was normalized by β-galactosidase activity in cell lysates, and expressed as an average of three independent experiments. The plasmids encoding for the dominant negative AMPK plasmids (DN-AMPK) and control plasmids (pcDNA) have been previously described (Lee *et al*., 2003).

Intracellular calcium measurements

HT-1080 cells cultured in 24-well plates (2×10^5 cells·mL^{-1}) in media were loaded with 5 μM Fluo-4-AM (Molecular Probes, Eugene, OR, USA), a Ca^{2+}-sensitive dye, for 45 min at 37°C. Following the pre-incubation, the cells were rinsed three times with media to remove any free dye and then incubated for 30 min in media alone to allow complete de-esterification of AM esters. Fluo-4-loaded cells were then stimulated with 5 mM metformin, 30 nM PMA, 10 μM BAPTA-AM or the vehicle DMSO. Changes in intracellular calcium were measured from captured fluorescence images of cells at 25 min, using the Axiovert 200 M Carl Zeiss fluorescence microscope (Carl Zeiss, Goettingen, Germany; excitation at 385 nm; emission at 512 nm).

Statistical analysis

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 when $P < 0.01$.

Materials

Metformin, BAPTA-AM, *N*-(6-amino-hexyl)-5-chloro-1-naphthalene sulphonamide (W7), EGTA and Gö6976 (Gö) were obtained from Calbiochem (La Jolla, CA, USA). PMA, compound C (AMPK inhibitor; AMPKi) and 5 - aminoimidazole - 4 - carboxamide - 1 - β - D - ribofuranoside (AICAR) were purchased from Sigma Chemical (St Louis, MO, USA). MTT-based colorimetric assay kit was purchased from Roche (Indianapolis, IN, USA). RPMI1640, FBS, sodium pyruvate and Trizol were supplied by Gibco BRL (Grand Island, NY, USA). Antibodies against phospho-MAP kinase, phospho-PKCα, phospho-CaMKI, 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. The MMP-9 promoter vector was kindly provided by Dr W Eberhardt (Klinikum der Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany) (Eberhardt *et al*., 2002). The pNF-κB-Luc and pAP-1-Luc reporter plasmids were obtained from Stratagene (La Jolla, CA, USA). The plasmids encoding for the dominant negative AMPK plasmids (DN-AMPK) and control plasmids (pcDNA) have been previously described (Lee *et al*., 2003). The other chemicals and reagents were of analytical grade.

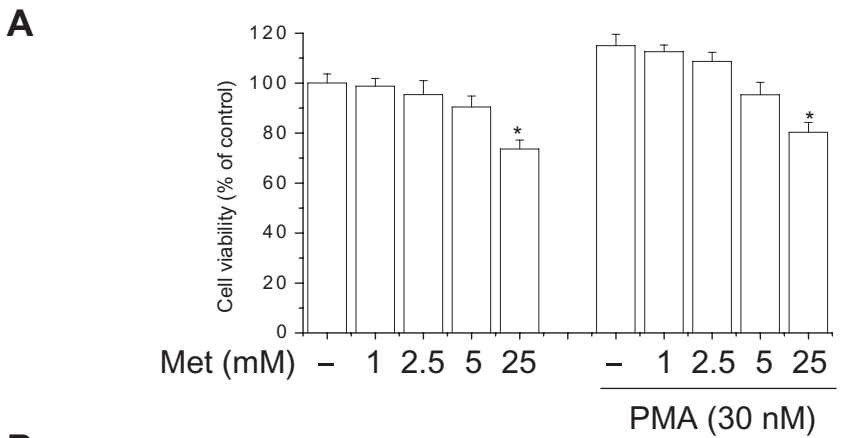
Results

Metformin prevents invasion and metastasis of human fibrosarcoma cells

Before investigating the pharmacological potential of metformin on PMA-induced MMP activity, we first determined the dose dependence of the cytotoxic effects of metformin in the absence or presence of PMA (30 nM) for 24 h in HT-1080 cells using the MTT assay. Metformin at concentrations lower than 5 mM had no cytotoxic effect on the cells, and metformin at 25 mM showed about a 20~28% decreases in cell viability in the absence or presence of PMA (Figure 1A). Thus, metformin had no significant cytotoxicity in tumour cells at these concentrations. *In vitro* invasion and migration assays, including transwell and wound-healing assays, were used to investigate the inhibitory effects of metformin on the invasive potency of fibrosarcoma HT-1080 cells. As illustrated in Figure 1B, the data from the wound-healing assay indicated that migration of HT-1080 cells was inhibited by metformin (Figure 1B). Similarly, the data obtained from the Matrigel invasion assay showed that PMA stimulated cell invasion, while 5 mM metformin inhibited the PMA-induced invasion of HT-1080 cells by 50% (Figure 1C). These results suggest that metformin prevents invasion and migration of human fibrosarcoma cells at non-toxic concentrations.

Metformin suppresses MMP-9 and -2 expressions

Metformin is known to activate AMP-activated protein kinase (AMPK). We examined the effect of metformin or AICAR, another AMPK activator, on MMP activity, which is related to the invasion and metastasis of fibrosarcoma. Treatment with metformin or AICAR inhibited the MMP-9 and -2 activities in a dose-dependent manner, as shown by gelatin zymography (Figure 2A,B). Furthermore, MMP-9 activity was strongly induced by PMA at 24 h, and metformin inhibited the activity of MMP-9 in a dose-dependent manner (Figure 2C). Metformin also inhibited the MMP-2 activity in a dose-dependent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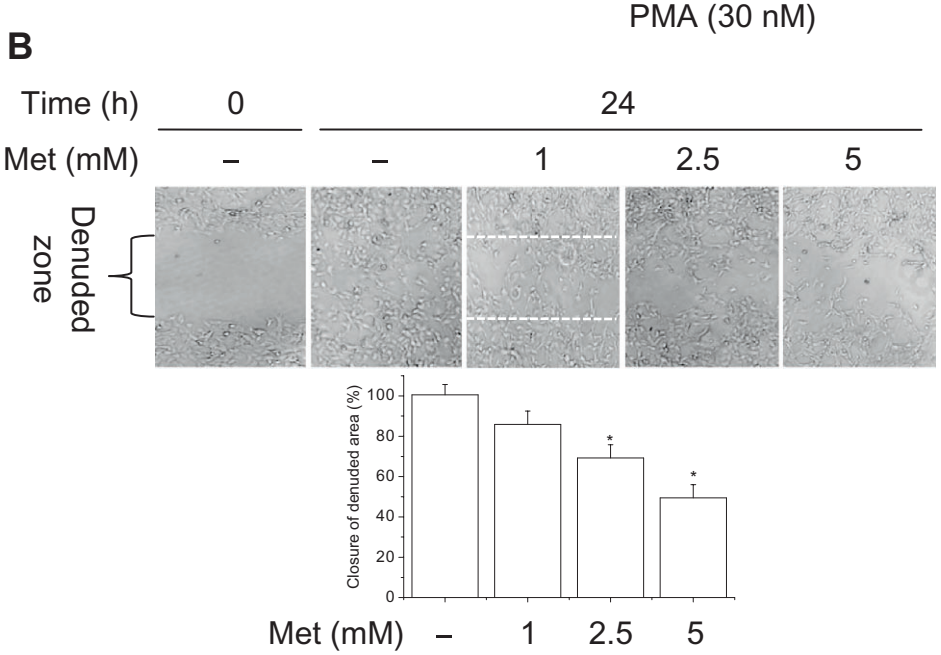
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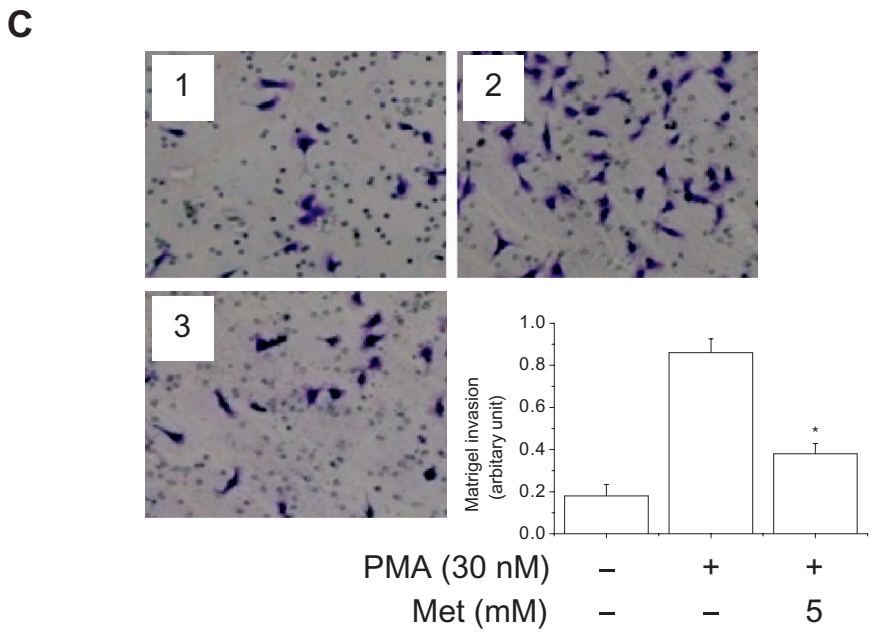
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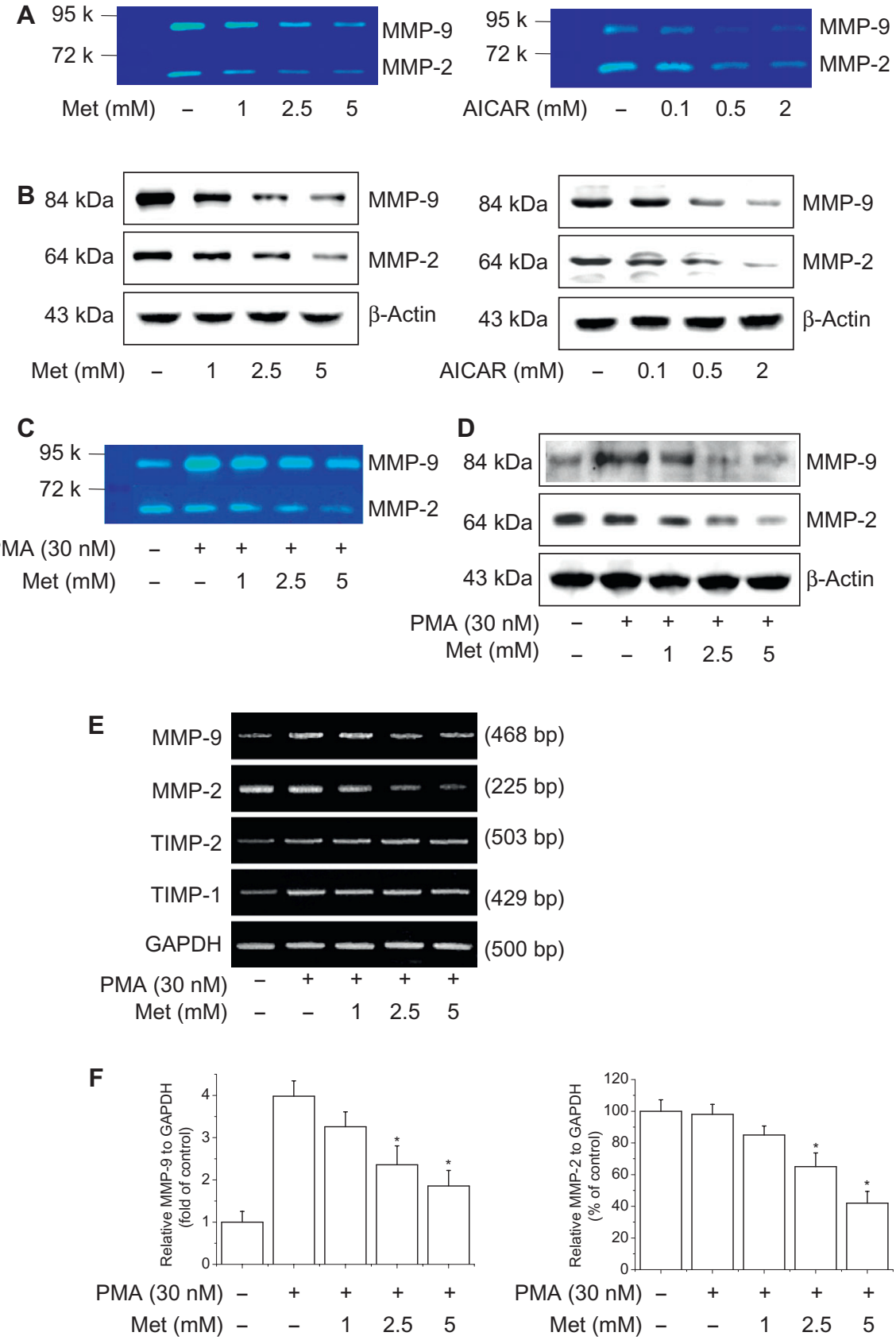
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GAPDH (500 bp)
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Met (mM) – – 1 2.5 5
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Relative MMP-9 to GAPDH (fold of control)
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PMA (30 nM) – + + + +
Met (mM) – – 1 2.5 5
Relative MMP-2 to GAPDH (% of control)
0 20 40 60 80 100 120
PMA (30 nM) – + + + +
Met (mM) – – 1 2.5 5

Figure 2 Inhibition of MMP-9 and -2 activities by metformin. (A) 80% Confluent HT-1080 cells were treated with various concentrations of metformin or AICAR in serum-free medium. The conditioned media were collected after 24 h, and gelatin zymography was performed. Each blot is representative of at least three others. (B) HT-1080 cells were incubated with metformin or AICAR for 24 h. MMP-9 and -2 expression in the media were analysed by Western blotting. β-Actin in the cell lysate is shown as a control. Each blot is representative of at least three others. (C) HT-1080 cells were incubated with varying concentrations of metformin in the presence of PMA (30 nM) for 24 h. MMP activity in the medium was analysed by gelatin zymography. Each blot is representative of at least three others. (D) HT-1080 cells were incubated with metformin and/or PMA (30 nM) for 24 h. MMP-9 and -2 expressions in the medium were analysed by Western blotting. β-Actin in the cell lysate is shown as a control. Each blot is representative of at least three others. (E and F) HT-1080 cells were incubated with metformin and/or PMA (30 nM) for 24 h. The mRNA expression of MMP-9, -2, TIMP-1 and TIMP-2 in the cells was analysed by semi-quantitative RT–PCR (E). GAPDH expression was included as an internal control. Each blot is representative of at least three others. MMP-9 and -2 mRNA expressions were analysed by real-time PCR (F). MMP-9 and -2 mRNA expressions were compared between treated and untreated cells at each time-point. *Significantly different from PMA treatment only ($P < 0.01$).

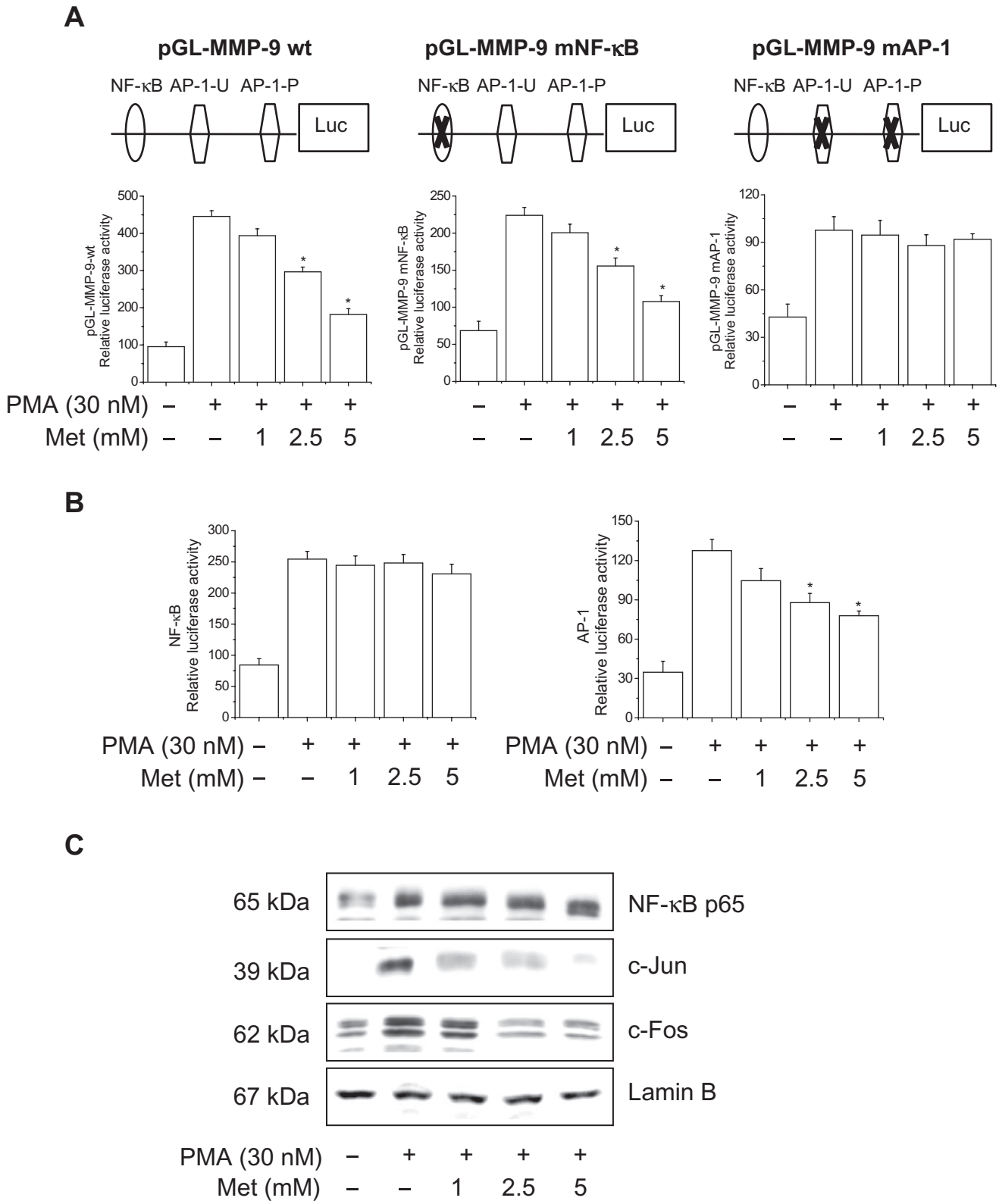
manner (Figure 2C). We studied the effect of metformin on the levels of MMP-9 and -2 mRNA in HT-1080 cells stimulated by PMA. Treatment of HT-1080 cells with metformin in the presence of PMA decreased the levels of MMP-9 and -2 mRNA, as evidenced by semi-quantitative RT–PCR or real-time PCR (Figure 2E,F). In addition, metformin blocked PMA-induced MMP-9 expression based on Western blot analysis (Figure 2D). Metformin also inhibited MMP-2 expression in a dose-dependent manner (Figure 2D). Because MMP-9 activity is tightly regulated by endogenous inhibitors, that is, tissue inhibitors of metalloproteinase (TIMPs) (Kazes *et al*., 2000), we further examined the expression level of TIMP-1 and -2 by semi-quantitative RT–PCR, but their expression remained essentially unchanged by treatment with metformin (Figure 2E). These results indicate that the lack of effect of metformin on RNA levels for TIMP-1 and -2 may also contribute to suppression of cell invasion.

Inhibition of transcriptional activity of MMP-9 gene through suppression of PMA-induced AP-1 activity by metformin

To investigate the importance of PMA and metformin in modulating MMP-9 expression, transient transfections were performed using human MMP-9 luciferase promoter constructs. Treatment with PMA led to a ~4.5-fold increase in MMP-9 promoter activity that was dose-dependently inhibited by metformin (Figure 3A). No cytotoxicity was observed when cells were exposed to metformin (data not shown). The MMP-9 promoter contains two important transcriptional elements, namely the binding sites of NF-κB and AP-1. To test which of these transcription factors might regulate the MMP-9 gene in HT-1080 cells, the 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 one or both AP-1 sites (Figure 3A). Treatment with metformin in the presence of PMA decreased the transcriptional activity of the reporter with the NF-κB mutation, but had no effect on the reporter with AP-1 mutations, suggesting that the target of metformin is the AP-1 transcription factor. In subsequent experiments, cells were transiently transfected with reporter vectors that included the tandem repeat of the NF-κB or AP-1 binding sites in order to confirm the specificity of the metformin-mediated inhibitory effect on AP-1. The luciferase activity in cells transfected with the AP-1 reporter was significantly and dose-dependently reduced by treatment with metformin (Figure 3B), whereas metformin had no statistically significant effect on the luciferase activity of cells transfected with the NF-κB reporters (Figure 3B). No cytotoxicity was observed when cells were exposed to metformin. These results clearly show that metformin regulated the transcriptional activation of MMP-9 through the inhibition of PMA-stimulated AP-1 activity. To investigate which of these transcription factors are involved in the inhibition of MMP-9 transcription by metformin in HT-1080 cells, we examined the effect of metformin on the PMA-stimulated nuclear translocation of p65, a major subunit of NF-κB, and c-Jun or c-Fos, major subunits of AP-1, which are required for the transcriptional activities. As shown in the Western blot analysis in Figure 3C, the protein levels of c-Jun and c-Fos in nuclear extracts were dose-dependently decreased by metformin treatment. However, metformin did not inhibit the PMA-induced nuclear translocation of p65. These data suggest that metformin regulates the transcriptional activation of MMP-9 through inhibition of PMA-stimulated AP-1 activity, but not NF-κB activity.

Metformin inhibits MMP-9 activation by an AMPK-independent mechanism

To elucidate whether the anti-invasion effect of metformin, in terms of down-regulation of MMP-9 activation, was associated with AMPK activation, we examined the phosphorylation of AMPK. We found that metformin induced phosphorylation of AMPKα in HT-1080 cells in a dose-dependent manner (Figure 4A). Next, we used compound C (AMPKi), an inhibitor of AMPK, whose ability to reverse the inhibitory effect of metformin indicates AMPK involvement. Here, HT-1080 cells were pretreated with AMPKi (1 μM) for 30 min and then treated with metformin (5 mM). Pretreatment with AMPKi indeed prevented metformin-mediated AMPK phosphorylation (Figure 4B), but did not reverse the suppressive effects of metformin on MMP-9 activation (Figure 4C–E). To further elucidate the role of AMPK in regulating MMP-9 inhibition, we tested the effect of AMPKi on AP-1 promoter activity. As shown in Figure 4F, AMPKi did not reverse the suppressive effect of metformin on AP-1 activity. No cytotoxicity was observed when cells were exposed to AMPKi (data not shown). This evidence suggests that the suppressive effect of metformin was not mediated through AMPK activation. We also tested 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), a known AMPK activator. Although AICAR (2 mM) induced AMPK phosphorylation in HT-1080 cells (Figure 4A), AMPKi did not reverse the suppressive effects of AICAR on MMP-9 expression (Figure 4C–F). HT-1080 cells were transfected with control



(pcDNA) plasmids or DN-AMPK prior to incubation with metformin or AICAR for 24 h, DN-AMPK did not reverse the suppressive effects of metformin or AICAR on MMP-9 expression (Figure 4G).

Metformin suppressed PMA-stimulated CaMKI, PKCα, ERK and JNK activation

Activation of one or more MAPK pathways is important for MMP-9 induction by PMA in various cell types (Woo *et al.*,

Figure 3 Inhibition of AP-1 activity in the MMP-9 promoter by metformin. 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 and pGL-MMP-9mAP-1 reporter plasmids (A), or with reporter plasmids containing tandem elements for NF-κB or AP-1 binding sites (B). Cells were cultured with metformin and/or PMA for 24 h, and the relative luciferase activity in the cell extract was determined. Data are expressed as the means ± SD of triplicate experiments. *Significantly different from PMA treatment only ($P < 0.01$). (C) Effects of metformin on PMA-induced c-Jun and c-Fos translocation. HT-1080 cells were pretreated with metformin (1–5 mM) for 1 h and then treated with 30 nM PMA for 3 h. Nuclear extracts were subjected to SDS–PAGE followed by Western blotting with anti-NF-κB, anti-c-Jun, anti-c-Fos and anti-lamin B antibodies. Each blot is representative of at least three others.

2004). To evaluate the effects of metformin on these signalling cascades, we used antibodies against the phosphorylated forms of the three MAPKs (i.e. ERK, JNK and p38). As shown in Figure 5A, their phosphorylation was increased by PMA stimulation. Metformin specifically decreased PMA-induced ERK1/2 and JNK1/2 phosphorylation, whereas the levels of phosphorylated p38 MAPK remained unchanged. These results suggest that metformin specifically suppresses ERK and JNK activity. Increased intracellular Ca^{2+} following PMA stimulation (Buys *et al*., 1984) is important as both a co-factor for the conventional PKC isoforms activated by PMA (Lin and Chen, 1998) and for activation of the Ca^{2+}/CaM pathway through binding to CaM (Soderling, 1999). CaM interacts with a wide array of kinases and phosphatases (Chin and Means, 2002), most notably the calmodulin kinase (CaMK) cascade. Next, we examined the effect of metformin on PMA-induced phosphorylation of CaMKI and PKCα, which are upstream modulators of MAPK pathways. While PMA activated CaMKI and PKCα phosphorylation, metformin significantly inhibited PMA-elicited CaMKI and PKCα phosphorylation (Figure 5B).

Metformin suppresses PMA-induced MMP-9 activation through Ca^{2+}-dependent PKC, ERK and JNK signalling pathways

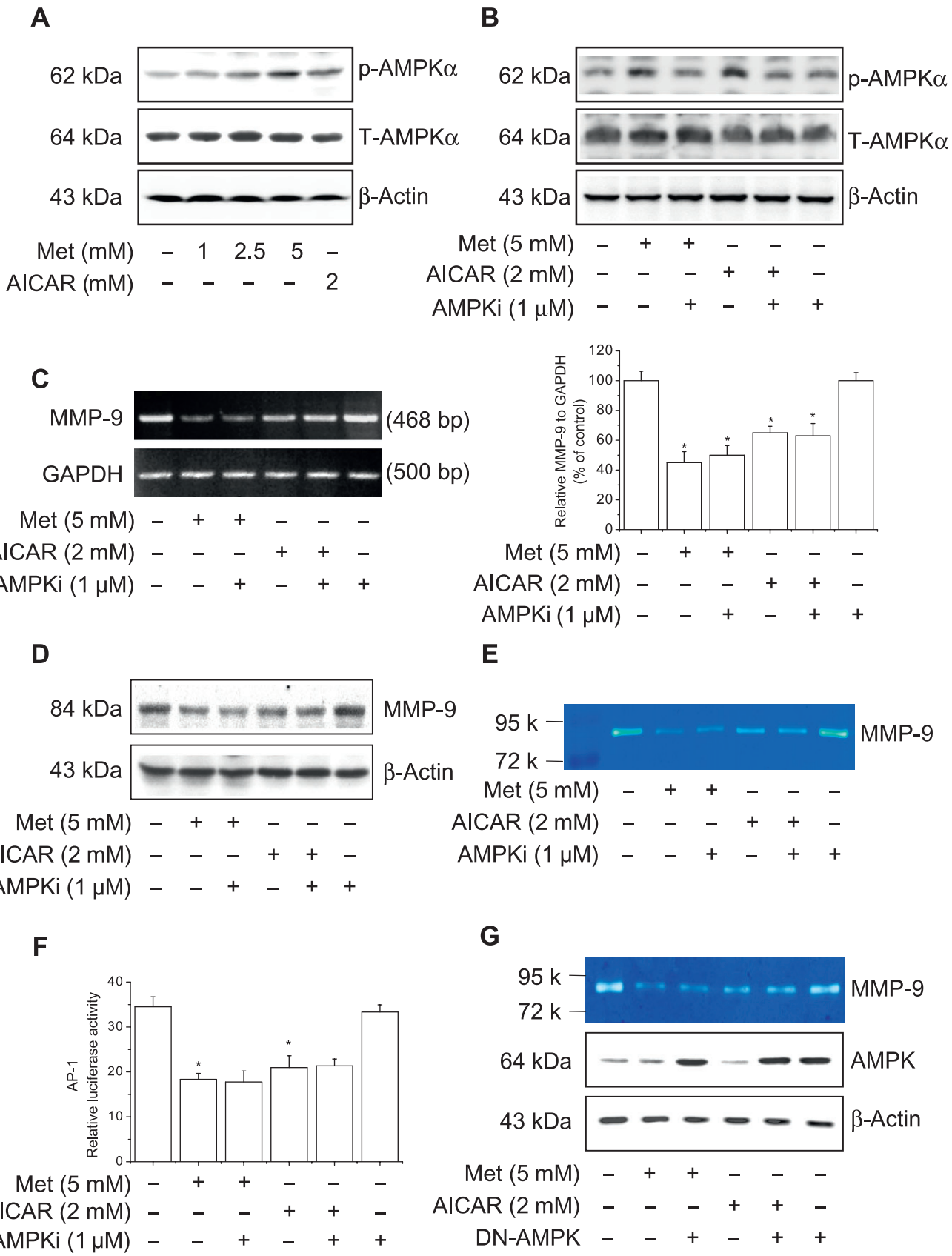
As reported previously, cytosolic Ca^{2+} is important for ERK activation, and MAPKs are differentially phosphorylated depending on the intra- or extracellular Ca^{2+} source (Mukhopadhyay *et al*., 2004). In addition, several studies have shown that PMA-induced MMP-9 activation is decreased by ERK1/2, p38 MAPK or JNK inhibitors or by PKC inhibitor (Lee *et al*., 2007). The subsequent experiments were designed to elucidate which of these signal transduction pathways is involved in PMA-stimulated MMP-9 expression and metformin inhibition of MMP-9 expression in HT-1080 cells. We investigated changes in Ca^{2+} in PMA-treated HT-1080 cells using the fluorescent indicator, Fluo-4-AM. PMA was added to HT-1080 cells that had been loaded with 5 μM Fluo-4-AM, and the cells were observed using fluorescence microscopy at 30 min (Figure 6A). PMA (30 nM) induced a transient increase in Ca^{2+} in HT-1080 cells. As shown in Figure 6A, pretreatment with metformin or the intracellular Ca^{2+} chelator BAPTA/AM (5 μM) or extracellular Ca^{2+} chelator EGTA (100 μM) completely blocked the PMA-induced transient increase in Ca^{2+} (Figure 6A). To evaluate the role of intracellular Ca^{2+} in PMA-mediated signalling, cells were first exposed to BAPTA-AM (an intracellular Ca^{2+} chelator) or W7 (Ca^{2+}/CaM antagonists) before PMA exposure. Cells treated with BAPTA-AM (10 μM) or W7 (40 μM) were then analysed for CaMKI, PKCα, ERK1/2 and JNK1/2 phosphorylation. As shown in Figure 6B, both BAPTA-AM and W7 modulated CaMKI, PKCα, ERK1/2 and JNK1/2 phosphorylation. As shown in Figure 6C, the protein levels of c-Jun and c-Fos in nuclear extracts were decreased by BAPTA-AM and W7 treatment (Figure 6C). Furthermore, in cells exposed to BAPTA-AM (10 μM) or W7 (40 μM), PMA-induced MMP-9 activation was significantly lower than in PMA-treated cells (Figure 6D,E). HT-1080 cells were transfected with CaMKI siRNA for 24 h, and then cells were exposed to PMA or vehicle (Figure 6F). MMP-9 expression was decreased by CaMKI siRNA treatment (Figure 6F).

Metformin suppresses invasion and migration through Ca^{2+}-dependent signalling pathways

In vitro invasion and migration assays were used to investigate the inhibitory effects of BAPTA-AM, W7 and Gö6976 (a calcium-dependent PKC inhibitor) on the invasive potency of fibrosarcoma HT-1080 cells. As illustrated in Figure 7A,B, the wound-healing assay and Matrigel invasion assay indicated that migration and invasion of HT-1080 cells were inhibited by intracellular Ca^{2+} chelator, Ca^{2+}/CaM antagonists or PKC inhibitor. These results suggest that metformin suppresses PMA-induced MMP-9 activation through Ca^{2+}-dependent PKC, ERK and JNK signalling pathways (Figure 8).

Discussion

Recent data suggest that metformin can inhibit *in vitro* migration of malignant glioma cells (Beckner *et al*., 2005) and inhibit breast and glial cancer cell proliferation *in vitro* (Isakovic *et al*., 2007; Ben Sahra *et al*., 2008; Hirsch *et al*., 2009; Phoenix *et al*., 2009). The role of the anti-diabetic drug metformin in glucose and fatty acid metabolism is well established. Metformin stimulates glucose uptake and increases fatty acid oxidation in muscle and liver with no side effects when administered at a dose of 1–3 mg per day. However, the mode of action and the biological consequences of this anti-diabetic drug in cancer cell migration and invasion are poorly understood. Metformin and AICAR have similar effects on metabolism, and both activate AMPK, which phosphorylates acetyl-coenzyme A carboxylase (ACC) (Zhou *et al*., 2001; Zang *et al*., 2004). The current study was designed to estimate the anti-invasive potential of metformin and to explore the molecular mechanisms underlying its activity. Our first experiments showed that metformin inhibited PMA-induced migration and invasion of HT-1080 cells. As described in previous studies (Cho *et al*., 2007), treatment with 2–30 nM PMA stimulated MMP-9


A
62 kDa
p-AMPKα
64 kDa
T-AMPKα
43 kDa
β-Actin
Met (mM) – 1 2.5 5 –
AICAR (mM) – – – – 2
B
62 kDa
p-AMPKα
64 kDa
T-AMPKα
43 kDa
β-Actin
Met (5 mM) – + + – – –
AICAR (2 mM) – – – + + –
AMPKi (1 μM) – – + – + +
C
MMP-9
(468 bp)
GAPDH
(500 bp)
Met (5 mM) – + + – – –
AICAR (2 mM) – – – + + –
AMPKi (1 μM) – – + – + +
Relative MMP-9 to GAPDH (% of control)
120
100
80
60
40
20
0
Met (5 mM) – + + – – –
AICAR (2 mM) – – – + + –
AMPKi (1 μM) – – + – + +
D
84 kDa
MMP-9
43 kDa
β-Actin
Met (5 mM) – + + – – –
AICAR (2 mM) – – – + + –
AMPKi (1 μM) – – + – + +
E
95 k
72 k
MMP-9
Met (5 mM) – + + – – –
AICAR (2 mM) – – – + + –
AMPKi (1 μM) – – + – + +
F
AP-1 Relative luciferase activity
40
30
20
10
0
Met (5 mM) – + + – – –
AICAR (2 mM) – – – + + –
AMPKi (1 μM) – – + – + +
G
95 k
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MMP-9
64 kDa
AMPK
43 kDa
β-Actin
Met (5 mM) – + + – – –
AICAR (2 mM) – – – + + –
DN-AMPK – – + – + +

Figure 4 Metformin activates AMPK, but does not affect PMA-induced MMP-9 activation. (A) After HT-1080 cells were treated with metformin or AICAR at the indicated concentrations, AMPK phosphorylation was examined by Western blotting. Each blot is representative of at least three others. (B) The suppressive effects of metformin and AICAR were independent of AMPK activation. Cells were pretreated with compound C (AMPKi; 1 μM) for 1 h prior to metformin or AICAR treatment, and the phosphorylation levels of AMPK was measured by Western blotting. Cells were pretreated for 1 h with compound C (AMPKi, 1 μM) followed by metformin or AICAR treatment for 24 h. Each blot is representative of at least three others. (C) The MMP-9 mRNA expression in the cells was analysed by semi-quantitative RT–PCR or real-time PCR. (D and E) Conditioned media were collected after 24 h, and then Western blotting or gelatin zymography was performed. Each blot is representative of at least three others. (F) Cells were transfected with pGL-AP-1 Luc reporter plasmids. The transfected cells were treated with compound C (AMPKi, 1 μM) for 1 h, and then metformin or AICAR for 24 h. The luciferase activity in the cell extract was determined. Data are expressed as the means ± SD of triplicate experiments. *Significantly different from control ($P < 0.01$). (G) Cells were transfected with DN-AMPK or pcDNA control for 24 h, and then cells were treated with metformin (5 mM) or AICAR (2 mM) for 24 h. Conditioned media were collected after 24 h, and then gelatin zymography was performed. AMPK expression in cell lysates was analysed by Western blotting. β-Actin in the cell lysates is shown as a control.

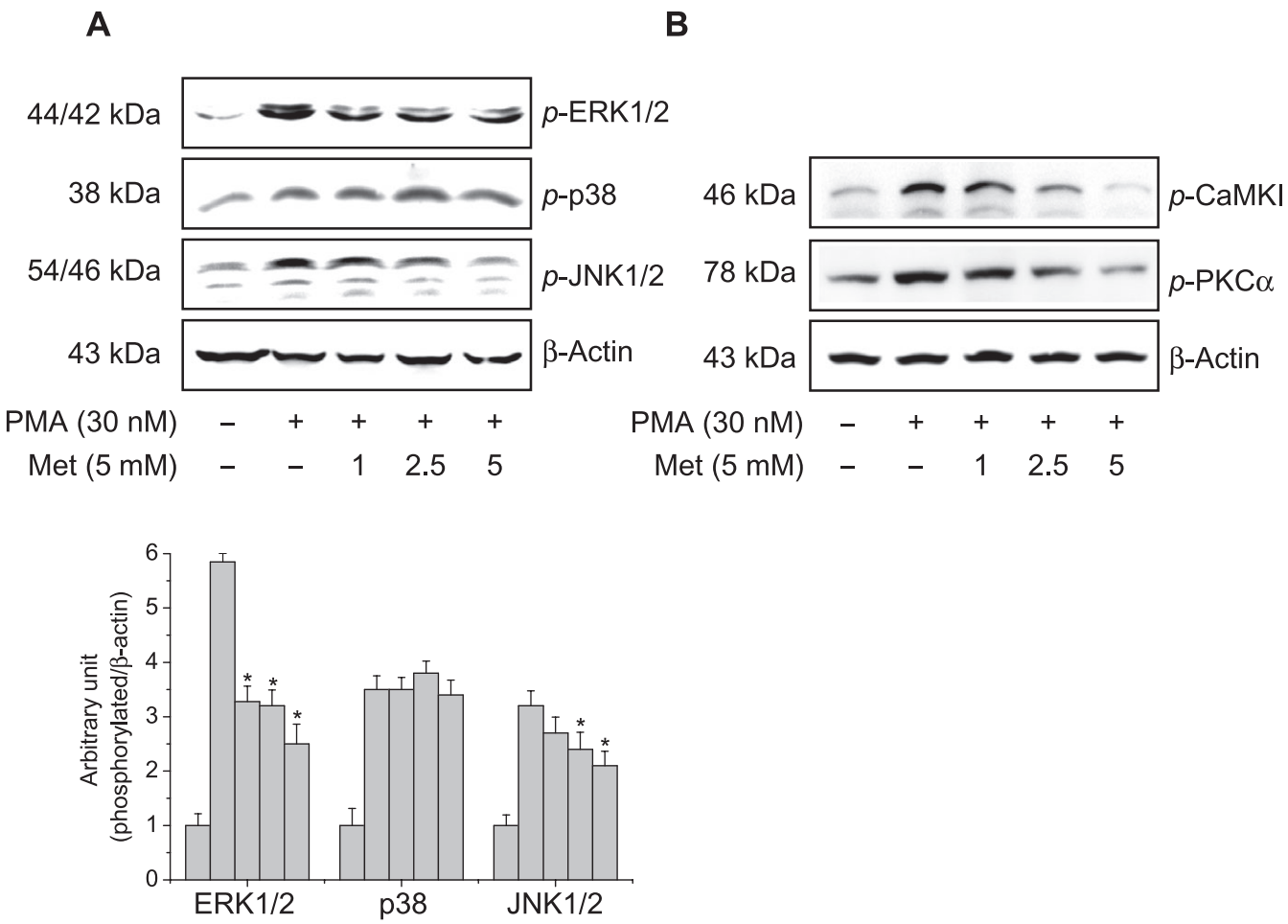
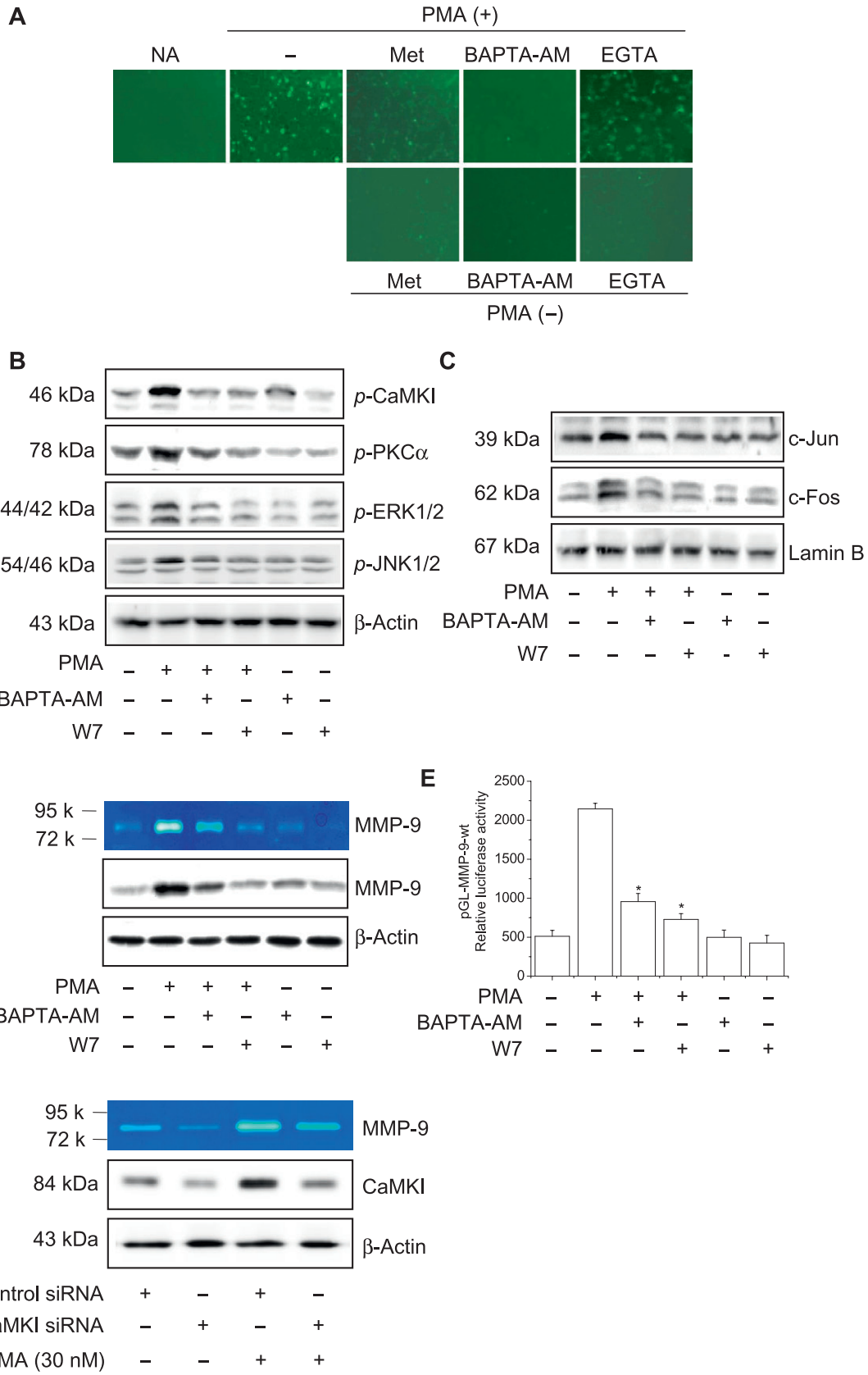


Figure 5 Effects of metformin on PMA-induced phosphorylation of MAPKs, CAMKI and PKCα. Cells were treated with PMA (30 nM) for 30 min in the presence or absence of metformin, and the phosphorylation levels of three MAPKs (A), CAMKI and PKCα (B) were measured by Western blotting. Each blot is representative of at least three others. Densitometry ratios of phospho-MAPKs were normalized to β-actin. Data are expressed as the means ± SD of triplicate experiments. *Significantly different from PMA treatment only ($P < 0.01$).

secretion in a dose-related manner (data not shown). In PMA-treated HT-1080 cells, metformin suppressed the increased expression of MMP-9 and -2.

We were further interested in the transcriptional mechanism of MMP-9 regulation by metformin. PMA enhances MMP-9 production through the activation of transcription factors, such as AP-1, NF-κB and SP-1 (Aggarwal *et al.*, 2004). Metformin suppressed MMP-9 induction by repressing the transcriptional activation of the MMP-9 promoter. Mutational analysis of the promoter revealed that the major target of metformin was AP-1, a finding that was further confirmed by the use of reporter plasmids containing synthetic elements that were specific for various transcription factors. We found that metformin inhibited the translocation of c-Jun and c-Fos, both of which are components of AP-1, to the nucleus in PMA-treated HT-1080 cells. These results clearly indicate that metformin inhibits MMP-9 activation by reducing the transcription factor, AP-1. Thus, it is evident that AP-1 is an


A
PMA (+)
NA
–
Met
BAPTA-AM
EGTA
Met
BAPTA-AM
EGTA
PMA (–)
B
46 kDa
p-CaMKI
78 kDa
p-PKCα
44/42 kDa
p-ERK1/2
54/46 kDa
p-JNK1/2
43 kDa
β-Actin
PMA – + + + – –
BAPTA-AM – – + – + –
W7 – – – + – +
C
39 kDa
c-Jun
62 kDa
c-Fos
67 kDa
Lamin B
PMA – + + + – –
BAPTA-AM – – + – + –
W7 – – – + - +
D
95 k
72 k
MMP-9
MMP-9
β-Actin
PMA – + + + – –
BAPTA-AM – – + – + –
W7 – – – + – +
E
pGL-MMP-9-wt
Relative luciferase activity
2500
2000
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1000
500
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PMA – + + + – –
BAPTA-AM – – + – + –
W7 – – – + – +
F
95 k
72 k
MMP-9
84 kDa
CaMKI
43 kDa
β-Actin
Control siRNA + – + –
CaMKI siRNA – + – +
PMA (30 nM) – – + +

Figure 6 Effect of metformin on intracellular Ca^{2+} concentration in HT-1080 cells. (A) Cells (2×10^5 cells·mL^{-1} n a 24-well microtitre plate) were treated with PMA (30 nM) for 30 min in the presence or absence of metformin, BAPTA-AM or EGTA, or 30 min at 37°C in a humidified incubator with a 5% CO_2 atmosphere. Cells were observed under a fluorescence microscope (excitation at 385 nm; emission at 512 nm). Each figure is representative of at least three others. (B) Effect of the calcium chelator BAPTA-AM or Ca^{2+}/CaM antagonist W7 on PMA-induced phosphorylation of CAMKI, PKCα, ERK and JNK in HT-1080 cells. Cells were treated with PMA (30 nM) for 30 min in the presence or absence of BAPTA-AM or W7, and the phosphorylation levels of CAMKI, PKCα, ERK and JNK were measured by Western blotting. Each blot is representative of at least three others. (C) Effect of the calcium chelator BAPTA-AM or Ca^{2+}/CaM antagonist W7 on PMA-induced c-Jun and c-Fos translocation in HT-1080 cells. Cells were pretreated with BAPTA-AM (10 μM) or W7 (40 μM) for 1 h, and treated with 30 nM PMA for 3 h. Nuclear extracts were subjected to SDS–PAGE followed by Western blotting with anti-c-Jun, anti-c-Fos and anti-lamin B antibodies. Each blot is representative of at least three others. (D and E) Effect of BAPTA-AM or W7 on PMA-induced MMP-9 activity in HT-1080 cells. Cells were pretreated with BAPTA-AM (10 μM) or W7 (40 μM) for 1 h, and treated with 30 nM PMA for 24 h. MMP-9 activity in the medium was analysed by gelatin zymography and Western blotting. Each blot is representative of at least three others. Cells were transfected with pGL-MMP-9WT reporter plasmids. The luciferase activity in the cell extract was determined. Data are expressed as the means ± SD of triplicate experiments. *Significantly different from PMA treatment only ($P < 0.01$). (F) Inhibition of PMA-induced MMP-9 expression by CaMKI siRNA transfection. Conditioned media were collected after 24 h, and then gelatin zymography. CaMKI expression in cell lysates was analysed by Western blotting. β-Actin in the cell lysate is shown as a control.

important transcriptional factor for MMP-9 expression, which in turn promotes cancer cell invasion, and metformin is likely to be a potent universal inhibitor of the activation of AP-1.

AMPK is a serine/threonine kinase and a member of the metabolite-sensing protein kinase family found in all eukaryotic cells (Zhou *et al.*, 2001). AMPK is a cellular fuel sensor pathway that is sensitive to increases in the AMP/ATP ratio. This pathway is a potent mediator of exercise-induced glucose uptake in skeletal muscle (Hardie, 2003). Once activated, AMPK phosphorylates and inactivates a number of metabolic enzymes involved in ATP-consuming cellular events such as fatty acid and protein synthesis involving ACC. Furthermore, AMPK activation has been shown to inhibit the mTOR pathway and S6K1 phosphorylation implicated in protein synthesis, suggesting that this pathway may regulate cell proliferation. Here, we showed that metformin inhibits MMP-9 expression in human fibrosarcoma HT-1080 cells, but mediates its effect independently of AMPK. Using compound C (AMPK inhibitor) or transfection with DN-AMPK, we showed that inhibition of the AMPK pathway did not reverse the effect of metformin on MMP-9 expression. Interestingly, other evidence indicates that metformin mediates its effects independently of AMPK. For example, Hue's group recently showed that the effects of metformin and AICAR on the function of glucokinase in hepatocytes are still observed in mice lacking α1 and α2 catalytic units (Guigas *et al.*, 2006). Ben Sahra *et al.* (2008) reported that metformin can exert AMPK-independent antitumoural effects in prostate cancer cells. Furthermore, Vazquez-Martin *et al.* reported that AMPK does not mediate all the anti-HER2 effects of metformin in breast cancer cells. Metformin has been extremely useful in studies of the AMPK pathway, but like any pharmacological tool, it may have other unknown functions independent of its initially described actions.

Several studies have identified signal transduction pathways that are involved in the regulation of MMP-9 expression in tumour cells (Simon *et al.*, 1998). The role of MAP kinases in the regulation of MMP-9 expression in malignant cells is especially well understood. In this study, we identified the signalling pathway-mediated regulation of the MMP-9 gene in PMA-induced HT-1080 cells in response to metformin treatment. Metformin suppressed PMA-induced phosphorylation of ERK and JNK, key pathways in PMA-induced cell invasion via MMP-9 expression (Figure 5A). These results demonstrate that metformin reduced MMP-9 expression by blocking AP-1 activation via ERK and JNK, and consequently inhibition of MMP9 production/activity and inhibition of migration/invasion in HT-1080 cells.

Alterations in Ca^{2+} are important for cell signalling. Ca^{2+} can stimulate the Ras/Raf/MEK/ERK signalling pathway via PKC, and the MEK/ERK pathway can be involved in cell migration (Yang *et al.*, 2003; He *et al.*, 2004). Changes in Ca^{2+} activate or inactivate target proteins, including PKCs, calpain and CaM. Increased intracellular Ca^{2+} following PMA stimulation (Buys *et al.*, 1984) is important as both a co-factor for the conventional PKC isoforms activated by PMA (Colbran and Soderling, 1990) and for activation of the Ca^{2+}/CaM pathway through binding to CaM (Rybalkin and Bornfeldt, 1999). Metformin completely blocked the PMA-induced increase in Ca^{2+} (Figure 6A). Furthermore, we observed that metformin down-regulated PMA-induced CaMKI and PKCα activities, as well as MMP-9 (Figure 7). In addition, the migration and invasion of HT-1080 cells were inhibited by BAPTA-AM (intracellular Ca^{2+} chelator), W7 (Ca^{2+}/CaM antagonists) or Gö6976 (a calcium-dependent PKC inhibitor).

It is well established that tumour cell migration and invasion depend on gelatinase activity. MMP-9 and MMP-2 are highly expressed in human cancer cells, and a direct relationship between cancer progression and gelatinase expression and activity has been well established in many studies (McCawley and Matrisian, 2000). We evaluated the inhibitory effect of MMP-9 siRNA on PMA-induced invasion in HT-1080 cells. As the results, the invasion of HT-1080 cells was decreased by MMP-9 siRNA treatment (Supporting Information Figure S1). As tumours manifest high levels of gelatinase activity, inhibitors specific for the gelatinases are urgently sought.

In summary, the present data show that metformin inhibited PMA-induced invasion and migration of human fibrosarcoma cells via Ca^{2+}-dependent PKCα/ERK and JNK/AP-1-signalling pathways. Metformin therefore has the potential to be a potent anti-cancer drug in therapeutic strategies for fibrosarcoma metastasis.

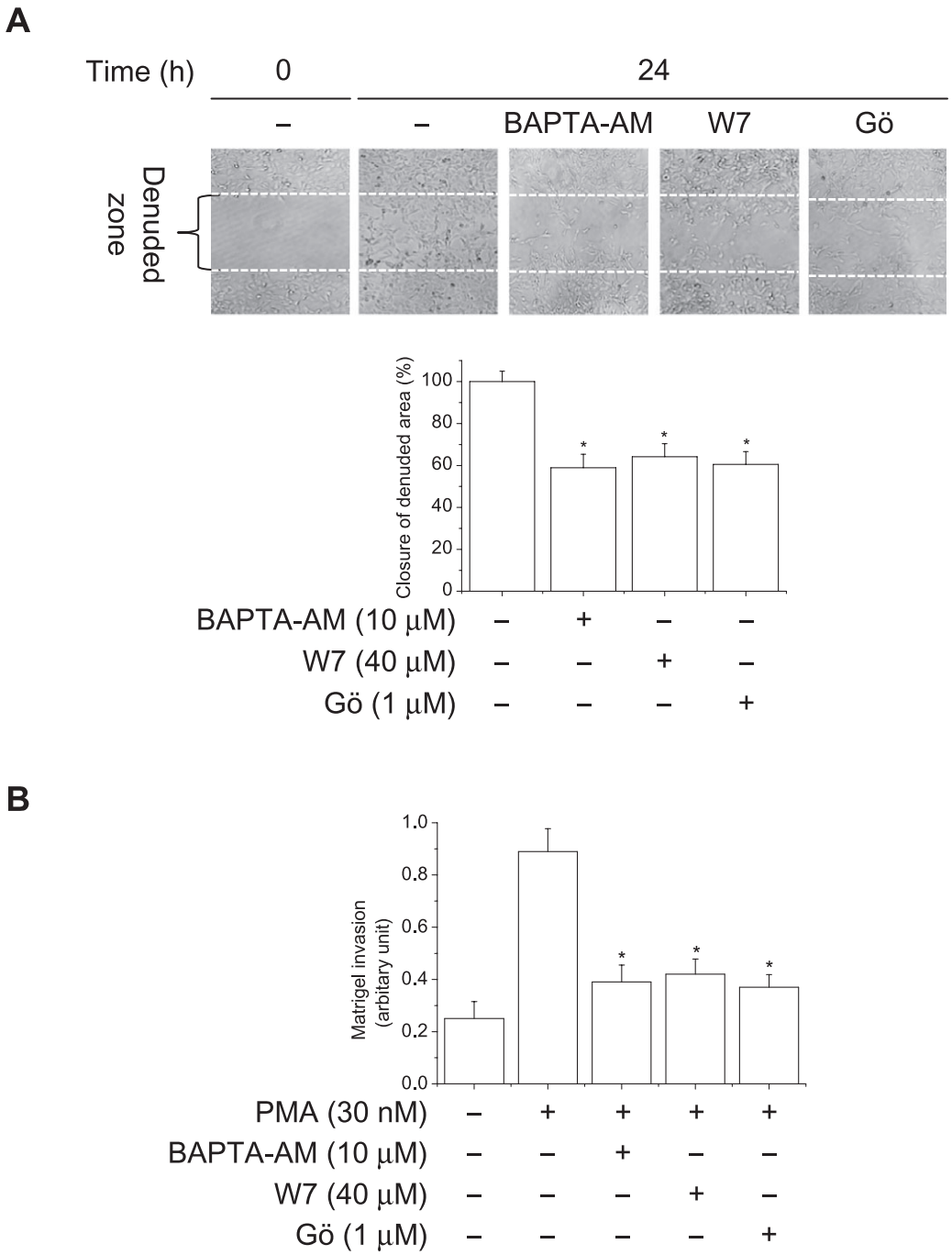

Figure 7 Effect of BAPTA-AM, W7 and Gö6976 on migration and invasion in HT-1080 cells. (A) Cell monolayers were scratched with a pipette tip and then treated with BAPTA-AM (10 μM), W7 (40 μM) and Gö6976 (a calcium-dependent PKC inhibitor; 1 μM) for 24 h. Migrating cells were photographed under phase contrast microscopy. Each figure is representative of at least three others. Data are expressed as the means ± SD of triplicate experiments. *Significantly different from control ($P < 0.01$). (B) Cells were pretreated with BAPTA-AM (10 μM), W7 (40 μM) and Gö6976 (1 μM) followed by PMA (30 nM) treatment for 24 h. After 24 h, cells on the bottom side of the filter were counted. Data are expressed as the means ± SD of triplicate experiments. *Significantly different from PMA treatment only ($P < 0.01$).

Acknowledgements

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Conflict of interest

None.

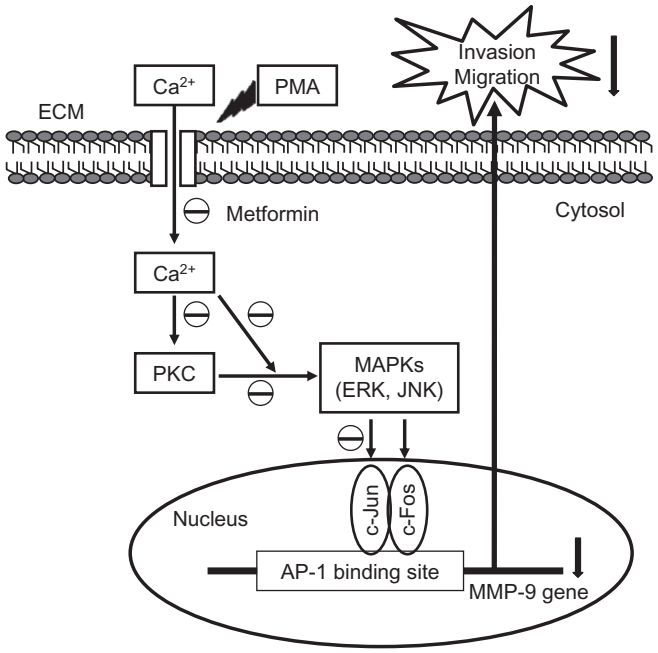

Figure 8 A model of the inhibitory effect of metformin in PMA-induced migration and invasion. This scheme delineates signalling steps identified in the present study. Metformin significantly suppressed MMP-9 activation by blocking the Ca^{2+} influx and the PKCα/ERK and JNK/AP-1-signalling pathways, and consequent reductions in migration and invasion of human fibrosarcoma cells were identified.

References

Aggarwal BB, Takada Y, Oommen OV (2004). From chemoprevention to chemotherapy: common targets and common goals. *Expert Opin Investig Drugs* **13**: 1327–1338.

Anisimov VN, Berstein LM, Egormin PA, Piskunova TS, Popovich IG, Zabezhinski MA *et al*. (2005). Effect of metformin on life span and on the development of spontaneous mammary tumors in HER-2/neu transgenic mice. *Exp Gerontol* **40**: 685–693.

Beckner ME, Gobbel GT, Abounader R, Burovic F, Agostino NR, Laterra J *et al*. (2005). Glycolytic glioma cells with active glycogen synthase are sensitive to PTEN and inhibitors of PI3K and gluconeogenesis. *Lab Invest* **85**: 1457–1470.

Ben Sahra I, Laurent K, Loubat A, Giorgetti-Peraldi S, Colosetti P, Auberger P *et al*. (2008). The antidiabetic drug metformin exerts an antitumoral effect *in vitro* and *in vivo* through a decrease of cyclin D1 level. *Oncogene* **27**: 3576–3586.

Bowker SL, Majumdar SR, Veugelers P, Johnson JA (2006). Increased cancer-related mortality for patients with type 2 diabetes who use sulfonylureas or insulin. *Diabetes Care* **29**: 254–258.

Buys SS, Keogh EA, Kaplan J (1984). Fusion of intracellular membrane pools with cell surfaces of macrophages stimulated by phorbol esters and calcium ionophores. *Cell* **38**: 569–576.

Buzzai M, Jones RG, Amaravadi RK, Lum JJ, DeBerardinis RJ, Zhao F *et al*. (2007). Systemic treatment with the antidiabetic drug metformin selectively impairs p53-deficient tumor cell growth. *Cancer Res* **67**: 6745–6752.

Catterall WA (2000). Structure and regulation of voltage-gated Ca^{2+} channels. *Annu Rev Cell Dev Biol* **16**: 521–555.

Cheung WY (2002). Calmodulin plays a pivotal role in cellular regulation. *Science* **207**: 19–27.

Chin D, Means AR (2002). Mechanisms for regulation of calmodulin kinase II alpha by Ca(2+)/calmodulin and autophosphorylation of threonine 286. *Biochemistry* **41**: 14001–14009.

Cho HJ, Kang JH, Kwak JY, Lee TS, Lee IS, Park NG *et al*. (2007). Ascofuranone suppresses PMA-mediated matrix metalloproteinase-9 gene activation through the Ras/Raf/MEK/ERK- and Ap1-dependent mechanisms. *Carcinogenesis* **28**: 1104–1110.

Colbran RJ, Soderling TR (1990). Calcium/calmodulin-dependent protein kinase II. *Curr Top Cell Regul* **31**: 181–221.

Deryugina EI, Luo GX, Reisfeld RA, Bourdon MA, Strongin A (1997). Tumor cell invasion through Matrigel is regulated by activated matrix metalloproteinase-2. *Anticancer Res* **17**: 3201–3210.

Dominguez LJ, Davidoff AJ, Srinivas PR, Standley PR, Walsh MF, Sowers JR (1996). Effects of metformin on tyrosine kinase activity, glucose transport, and intracellular calcium in rat vascular smooth muscle. *Endocrinology* **137**: 113–121.

Eberhardt W, Schulze M, Engels C, Klasmeier E, Pfeilschifter J (2002). 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* **16**: 1752–1766.

Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD (2005). Metformin and reduced risk of cancer in diabetic patients. *BMJ* **330**: 1304–1305.

Gossas T, Danielson UH (2006). Characterization of Ca2þ interactions with matrix metallopeptidase-12: implications for matrix metallopeptidase regulation. *Biochem J* **398**: 393–398.

Guigas B, Bertrand L, Taleux N, Foretz M, Wiernsperger N, Vertommen D *et al*. (2006). 5-Aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside and metformin inhibit hepatic glucose phosphorylation by an AMP-activated protein kinase-independent effect on glucokinase translocation. *Diabetes* **55**: 865–874.

Hait WN, Lazo JS (1986). Calmodulin: a potential target for cancer chemotherapeutic agents. *J Clin Oncol* **4**: 994–1012.

Hardie DG (2003). Minireview: the AMP-activated protein kinase cascade: the key sensor of cellular energy status. *Endocrinology* **144**: 5179–5183.

He YY, Huang JL, Chignell C (2004). Delayed and sustained activation of extracellular signal-regulated kinase in human keratinocytes by UVA. *J Biol Chem* **279**: 53867–53874.

Hirsch HA, Iliopoulos D, Tsichlis PN, Struhl K (2009). Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. *Cancer Res* **69**: 7507–7511.

Isakovic A, Harhaji L, Stevanovic D, Markovic Z, Sumarac-Dumanovic M, Starcevic V *et al*. (2007). Dual antiglioma action of metformin: cell cycle arrest and mitochondria-dependent apoptosis. *Cell Mol Life Sci* **64**: 1290–1302.

Johnsen M, Lund LR, Romer J, Almholt K, Dano K (1998). Cancer invasion and tissue remodeling: common themes in proteolytic matrix degradation. *Curr Opin Cell Biol* **10**: 667–671.

Johnson LL, Dyer R, Hupe DJ (1998). Matrix metalloproteinases. *Curr Opin Chem Biol* **2**: 466–471.

Johnson MR, Wang K, Smith JB, Heslin MJ, Diasio RB (2000). Quantitation of dihydropyrimidine dehydrogenase expression by real-time reverse transcription polymerase chain reaction. *Anal Biochem* **278**: 175–184.

Kajanne R, Miettinen P, Mehlem A, Leivonen SK, Birrer M, Foschi M *et al*. (2007). EGF-R regulates MMP function in fibroblasts through MAPK and AP-1 pathways. *J Cell Physiol* **212**: 489–497.

Kazes I, Elalamy I, Sraer JD, Hatmi M, Nguyen G (2000). Platelet release of trimolecular complex components MT1-MMP/TIMP2/MMP2: involvement in MMP2 activation and platelet aggregation. *Blood* **96**: 3064–3069.

Kim DS, Suh KH (2001). Cell cycle-dependent activity change of Ca^{2+}/calmodulin-dependent protein kinase II in NIH 3T3 cells. *J Biochem Mol Biol* **34**: 212–218.

Klee CB (1991). Concerted regulation of protein phosphorylation and dephosphorylation by calmodulin. *Neurochem Res* **16**: 1059–1065.

Kobayashi T, Kishimoto J, Ge Y, Jin W, Hudson DL, Ouahes N *et al*. (2001). A novel mechanism of matrix metalloproteinase-9 gene expression implies a role for keratinization. *EMBO Rep* **2**: 604–608.

Kohn EC, Jacobs W, Kim YS, Alessandro R, Stetler-Stevenson WG, Liotta LA (1994). Calcium influx modulates expression of matrix metalloproteinase-2 (72-kDa type IV collagenase, gelatinase A). *J Biol Chem* **269**: 21505–21511.

Lee M, Hwang JT, Lee HJ, Jung SN, Kang I, Chi SG *et al*. (2003). AMP-activated protein kinase activity is critical for hypoxia-inducible factor-1 transcriptional activity and its target gene expression under hypoxic conditions in DU145 cells. *J Biol Chem* **278**: 39653–39661.

Lee SO, Jeong YJ, Im HG, Kim CH, Chang YC, Lee IS (2007). Silibinin suppresses PMA-induced MMP-9 expression by blocking the AP-1 activation via MAPK signaling pathways in MCF-7 human breast carcinoma cells. *Biochem Biophys Res Commun* **354**: 165–171.

Lin WW, Chen B (1998). Distinct PKC isoforms mediate the activation of cPLA2 and adenylyl cyclase by phorbol ester in RAW264.7 macrophages. *Br J Pharmacol* **125**: 1601–1609.

Liotta LA, Steeg PS, Stetler-Stevenson WG (1991). Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell* **64**: 327–336.

McCawley LJ, Matrisian LM (2000). Matrix metalloproteinases: multifunctional contributors to tumor progression. *Mol Med Today* **6**: 149–156.

Mercure MZ, Ginnan R, Singer HA (2008). CaM kinase II delta2-dependent regulation of vascular smooth muscle cell polarization and migration. *Am J Physiol Cell Physiol* **294**: C1465–C1475.

Mukhopadhyay S, Munshi HG, Kambhampati S, Sassano A, Platanias LC, Stack MS (2004). Calcium-induced matrix metalloproteinase 9 gene expression is differentially regulated by ERK1/2 and p38 MAPK in oral keratinocytes and oral squamous cell carcinoma. *J Biol Chem* **279**: 33139–33146.

Phoenix KN, Vumbaca F, Claffey KP (2009). Therapeutic metformin/AMPK activation promotes the angiogenic phenotype in the ERalpha negative MDA-MB-435 breast cancer model. *Breast Cancer Res Treat* **113**: 101–111.

Roderick HL, Cook SJ (2008). Ca^{2+} signalling checkpoints in cancer: remodelling Ca^{2+} for cancer cell proliferation and survival. *Nat Rev Cancer* **8**: 361–375.

Rybalkin SD, Bornfeldt KE (1999). Cyclic nucleotide phosphodiesterases and human arterial smooth muscle cell proliferation. *Thromb Haemost* **82**: 424–434.

Salo T, Mäkelä M, Kylmäniemi M, Autio-Harmainen H, Larjava H (1994). Expression of matrix metalloproteinase-2 and -9 during early human wound healing. *Lab Invest* **70**: 176–182.

Sato T, Koike L, Miyata Y, Hirata M, Mimaki Y, Sashida Y *et al*. (2002). Inhibition of activator protein-1 binding activity and phosphatidylinositol 3-kinase pathway by nobiletin, a polymethoxy flavonoid, results in augmentation of tissue inhibitor of metalloproteinases-1 production and suppression of production of matrix metalloproteinases-1 and -9 in human fibrosarcoma HT-1080 cells. *Cancer Res* **62**: 1025–1029.

Schmidt HH, Pollock JS, Nakane M, Forstermann U, Murad F (1992). Ca^{2+}/calmodulin-regulated nitric oxide synthases. *Cell Calcium* **13**: 427–434.

Schneider MB, Matsuzaki H, Haorah J, Ulrich A, Standop J, Ding XZ *et al*. (2001). Prevention of pancreatic cancer induction in hamsters by metformin. *Gastroenterology* **120**: 1263–1270.

Schuller HM, Correa E, Orloff M, Reznik GK (1990). Successful chemotherapy of experimental neuroendocrine lung tumors in hamsters with an antagonist of Ca^{2+}/calmodulin. *Cancer Res* **50**: 1645–1649.

Sharma RV, Bhalla RC (1995). Metformin attenuates agonist-stimulated calcium transients in vascular smooth muscle cells. *Clin Exp Hypertens* **17**: 913–929.

Shim JS, Lee J, Kim KN, Kwon HJ (2007). Development of a new Ca^{2+}/calmodulin antagonist and its anti-proliferative activity against colorectal cancer cells. *Biochem Biophys Res Commun* **359**: 747–751.

Shin Y, Yoon SH, Choe EY, Cho SH, Woo CH, Rho JY *et al*. (2007). PMA-induced up-regulation of MMP-9 is regulated by a PKCalpha-NF-kappaB cascade in human lung epithelial cells. *Exp Mol Med* **39**: 97–105.

Simon C, Goepfert H, Boyd D (1998). Inhibition of the p38 mitogen activated protein kinase by SB 203580 blocks PMA-induced Mr 92 000 type IV collagenase secretion and *in vitro* invasion. *Cancer Res* **58**: 1135–1139.

Soderling TR (1999). The Ca-calmodulin-dependent protein kinase cascade. *Trends Biochem Sci* **24**: 232–236.

Strobl JS, Peterson VA (1992). Tamoxifen-resistant human breast cancer cell growth: inhibition by thioridazine, pimozide and the calmodulin antagonist, W-13. *J Pharmacol Exp Ther* **263**: 186–193.

Sudbeck BD, Pilcher BK, Pentland AP, Parks WC (1997). Modulation of intracellular calcium levels inhibits secretion of collagenase 1 by migrating keratinocytes. *Mol Biol Cell* **8**: 811–824.

Walsh MP, Vallet B, Autric F, Demaille JG (1979). Purification and characterization of bovine cardiac calmodulin-dependent myosin light chain kinase. *J Biol Chem* **254**: 2136–2144.

Wei JW, Morris HP, Hickie RA (1982). Positive correlation between calmodulin content and hepatoma growth rates. *Cancer Res* **42**: 571–574.

Westermarck J, Kahari VM (1999). Regulation of matrix metalloproteinase expression in tumor invasion. *FASEB J* **13**: 781–792.

Woo JH, Lim JH, Kim YH, Suh SI, Min DS, Chang JS *et al*. (2004). Resveratrol inhibits phorbol myristate acetate induced matrix metalloproteinase-9 expression by inhibiting JNK and PKC delta signal transduction. *Oncogene* **23**: 1845–1853.

Yan C, Boyd DD (2007). Regulation of matrix metalloproteinase gene expression. *J Cell Physiol* **211**: 19–26.

Yang L, Ng D, Bikle D (2003). Role of protein kinase C α in calcium induced keratinocyte differentiation: defective regulation in squamous cell carcinoma. *J Cell Physiol* **195**: 249–259.

Zang M, Zuccollo A, Hou X, Nagata D, Walsh K, Herscovitz H *et al*. (2004). AMP-activated protein kinase is required for the lipid-lowering effect of metformin in insulin-resistant human HepG2 cells. *J Biol Chem* **279**: 47898–47905.

Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J *et al*. (2001). Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* **108**: 1167–1174.

Supporting information

Additional Supporting Information may be found in the online version of this article.

Figure S1 Effect of MMP-9 siRNA on PMA-induced invasion of HT-1080 cells. Cells were transfected with MMP-9 siRNA or control siRNA for 24 h, and then cells were treated with PMA (30 nM) or vehicle for 24 h. (A) Conditioned media were collected after 24 h, and then gelatin zymography was performed. MMP-9 mRNA expression in the cells was analysed by semi-quantitative RT–PCR. GAPDH expression was included as an internal control. (B) After treatment with PMA for 24 h, cells on the bottom side of the filter

were counted. Data are expressed as the means ± SD of triplicate experiments. *Significantly different from control ($P < 0.01$). **Significantly different from PMA treatment only ($P < 0.01$).