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ORIGINAL ARTICLE

Metformin inhibits the invasion of human hepatocellular carcinoma cells and enhances the chemosensitivity to sorafenib through a downregulation of the ERK/JNK-mediated NF-κB-dependent pathway that reduces uPA and MMP-9 expression

Shu-Ching Hsieh · Jen-Pi Tsai · Shun-Fa Yang · Meng-Ju Tang · Yi-Hsien Hsieh

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Abstract Metformin has been shown to exert anti-cancer activities in several cancer cells and animal models. However, the molecular mechanisms of its anti-metastatic activities remain poorly understood and warrant further investigation. The aims of this study were to evaluate the ability of metformin to inhibit the migration and invasion of hepatocellular carcinoma (HCC) cells and identify its effects on signaling pathways. Our data indicate that metformin inhibits the migration and invasion of human HCC cells. Metformin was also found to significantly inhibit the expression and secretion of MMP-9 and uPA in HCC cells, and suppress the phosphorylation of ERK1/2 and JNK1/2. Treatment with an ERK1/2 inhibitor (PD98059) or JNK1/2 inhibitor (SP600125) enhanced the inhibitory effects of metformin on the migration and invasion of HCC cells. Moreover, metformin-induced inhibition of MMP-9 and uPA promoter activity also blocked the nuclear translocation of NF-κB and its binding to the MMP-9 and uPA promoters, and these suppressive effects were further enhanced by PD98059 or SP600125. Moreover, metformin markedly enhanced the anti-metastatic effects of sorafenib. In conclusion, metformin inhibits the migration and invasion of HCC cells by suppressing the ERK/JNK-mediated NF-κB-dependent pathway, and thereby reducing uPA and MMP-9 expression. Additionally, combination treatment with metformin and sorafenib yielded synergistic inhibitory effects in suppressing cell migration and invasion of HCC cells. These findings provide insight into the molecular mechanisms involved in the anti-metastatic effects of metformin, as well as its ability to enhance the chemosensitivity of HCC cells to sorafenib.

Keywords Hepatocellular carcinoma cells · Metformin · Invasion · Migration · MMP-9 · uPA · Sorafenib

Abbreviations

ChIP	Chromatin immunoprecipitation
HCC	Hepatocellular carcinoma
MMP-9	Matrix metalloproteinase-9
qRT-PCR	Quantification reverse transcriptase-polymerase chain reaction
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NF-κB	Nuclear factor-κB
uPA	Urokinase-type plasminogen activator

Introduction

Hepatocellular cell carcinoma (HCC) is an aggressive malignant tumor. Studies have reported that the

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development of HCC could be caused by viral hepatitis, alcoholic liver disease, and nonalcoholic fatty liver disease (El-Serag and Rudolph 2007). Recently, diabetes mellitus (DM) was found to be an independent risk factor for HCC (Giovannucci et al. 2010). Following a diagnosis of HCC, distant metastasis is an importance index of prognosis (Nabeshima et al. 2002; Okita 2006). Tumor metastasis involves a complex cascade of events, including cell adhesion, invasion, and angiogenesis. Matrix metalloproteinase (MMP) and urokinase-type plasminogen activator (uPA) systems, which degrade the basement membrane and extracellular matrix, are two of the most crucial proteolytic proteinases that facilitate the invasion of malignant cells (Choong and Nadesapillai 2003; Liotta and Stetler-Stevenson 1991). An upregulation of MMP and uPA expression and activity has been shown to play a key role in many human cancers with invasive and metastatic capability (Mook et al. 2004). In HCC patients with portal cancer emboli, tumor invasion, and metastasis, an overexpression of uPA, the uPA receptor, and plasminogen-activator inhibitor type-1 have been previously reported (Blasi and Sidenius 2010).

Several studies have shown that anti-diabetic medications may modify the risk of metastasis. In addition to being a widely prescribed oral anti-diabetic drug in the treatment of type 2 DM (Kahn et al. 2005) and having beneficial effects on hepatic glucose production and reduction of insulin resistance in peripheral tissue leading to enhanced glucose uptake and utilization in skeletal muscle (Li et al. 2011), metformin has been recently shown to be associated with a risk reduction against human HCC (Chen et al. 2013a; Singh et al. 2013). Additionally, some studies revealed that metformin has the ability to inhibit the in vitro migration of highly malignant glioma cells (Beckner et al. 2005), as well as PMA-induced migration and invasion of human fibrosarcoma HT-1080 cells through the PKCα/ERK and JNK/AP-1 signaling pathways (Hwang and Jeong 2010). In vivo studies revealed that metformin decreases the incidence and size of mammary adenocarcinomas in Her2/Neu mice (Cufi et al. 2012) and suppresses tumor growth of HCT116 colon cells in a mouse xenograft model (Buzzai et al. 2007). These results suggest that metformin may be used as an anti-cancer therapy. However, the mechanisms of action by which metformin inhibits human HCC cell migration and invasion are not well understood.

According to the EASL-EORTC guidelines, therapeutic approaches for human HCC are potentially curative, palliative, and symptomatic, and the choice of treatment is based on the stage of HCC. Since HCC is often diagnosed at an advanced stage when curative therapies, such as liver transplantation or surgical resection are of limited efficacy, oral sorafenib treatment has been used for advanced-stage HCC (Cheng et al. 2009). Sorafenib, an oral multi-kinase inhibitor with activities against Raf serine/threonine kinases, vascular endothelial growth factor receptor, and platelet-derived growth factor receptor tyrosine kinase, has a potent anti-angiogenic and pro-apoptotic activity, and therefore presents a marked anti-tumoral effect. According to two international randomized controlled trial, the SHARP (Sorafenib HCC Assessment Randomized Protocol) and the Asia-Pacific trials, sorafenib was confirmed to improve the median overall survival and median time to radiological progression in advanced HCC patients (Llovet et al. 2008). Although sorafenib has a positive effect on the survival of advanced HCC patients, the response to sorafenib remains low and the median overall survival is only extended by 2.8 months (Cheng et al. 2009). Accordingly, the therapeutic approach for the vast majority of advanced-stage HCC patients needs to be enhanced.

In recent years, despite encouraging reports on the efficacy of anti-viral therapy for viral hepatitis, as well as the surveillance and treatment of HCC, the prognosis for advanced-stage HCC remains dismal. Since it is important to inhibit the spread of tumor cells, and thereby metastasis, and little is known about the molecular mechanisms responsible for the anti-metastatic properties of metformin with and without sorafenib in inhibiting human HCC cells, this study aimed to clarify the mechanisms of metformin monotherapy and combination therapy with sorafenib on highly invasive human HCC cells.

Materials and methods

Reagents

Metformin of 99 % purity was purchased from Sigma (St. Louis, MO). A stock solution of metformin was made at a concentration of 1 M in PBS and stored at −20 °C. Antibodies against MMP-9, uPA, p-ERK1/2, p-p38, p-JNK, p-Akt, ERK1/2, p38, JNK1/2, Akt, NF-κB, (p65), lamin B, α-tubulin, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-labeled anti-mouse and anti-rabbit secondary antibodies were obtained from Promega (Madison, WI). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] was purchased from Sigma (St. Louis, MO). The MEK1/2 inhibitor, PD98059, JNK1/2 inhibitor, SP600125, and sorafenib were purchased from Calbiochem (San Diego, CA). All stock solutions were wrapped in foil and kept at −20 °C.

Cell culture

HA22T/VGH cells (BCRC No. 60168) was purchased from the Bioresources Collection and Research Center, Food Industry Research and Development Institute (Hsinchu,

Taiwan), SK-Hep-1 cells (ATCC HTB-52) from the American Type Culture Collection (Rockville, MD) and HuH-7 cells from the Riken Cell Bank (Tsukuba, Japan) were grown in DMEM medium supplemented with 10 % fetal bovine serum (Hyclone Laboratories, Logan, UT, USA), 2 mg/ml glutamine, 100 μg/ml NEAA, 100 mg/ml penicillin–streptomycin. The primary normal liver THLE-2 cells (ATCC CRL-2706) from the American Type Culture Collection (Rockville, MD) were grown in BEGM kit medium (Lonza/Clonetics Corporation, Walkersville) supplemented with gentamycin/amphotericin (GA) and epinephrine and to which we add extra 5 ng/mL EGF, 70 ng/mL phosphoethanolamine, 10 % fetal bovine serum and 100 mg/ml penicillin–streptomycin. The cells were maintained under standard cell culture conditions at 37 °C and 5 % CO_2 in a humid environment.

Cell proliferation assay

Cell proliferation was assessed by using an MTT assay (Yu et al. 2012). Cells were plated into 24-well plates at 4 × 104 cells/well, grown for 24 h, and treated with different concentrations of metformin (0, 10, 30, and 50 mM). After a 24-h incubation, MTT reagent was added to each well until a final concentration of 0.5 mg/mL was achieved, and the mixture of MTT and cells were further incubated for 4 h. The viable cell number was directly proportional to the production of formazan following solubilization with isopropanol. Color intensity was measured at 570 nm in a Multiskan MS ELISA reader (Labsystems, Helsinki, Finland). All experiments were performed in triplicate.

Migration and invasion assay

The migration assay was performed using a 48-well Boyden chamber plate (Neuro Probe) with 8-μm pore size polycarbonate membrane filters, as previously described (Hsia et al. 2013). The lower compartment was filled with DMEM containing 20 % FBS. HA22T/VGH, SK-Hep-1 and Huh-7 cells were placed in the upper part of the Boyden chamber and incubated for 8 and 12 h, respectively. The invasion assay was done using a 48-well Boyden chamber with polycarbonate filters. The upper side was pre-coated with 10 μg/mL Matrigel (Collaborative Biomedical Products). HA22T/VGH, SK-Hep-1 and Huh-7 cells were placed in the upper part of the Boyden chamber and incubated at 37 °C for 12 and 24 h, respectively. After incubating, cells were fixed with methanol and stained with 0.05 % Giemsa for 1 h. Cells on the upper surface of the filter were removed with a cotton swab. The filters were then rinsed with distilled water until no additional stain was leached out. Cells were then air dried for 20 min. Migratory phenotypes were determined by counting the number of cells that migrated to the lower side of the filter using microscopy. The fourth fields were counted for each filter, and each sample was assayed in triplicate.

Analysis of MMP-9 and uPA activities (zymography assay)

The activities of MMP-9 were assayed by gelatin zymography, as described previously (Tung et al. 2013). Briefly, conditioned media from cells cultured in the absence of serum for 24 h were collected. Samples were mixed with loading buffer and electrophoresed on 8 % SDS-polyacrylamide gel containing 0.1 % gelatin. Electrophoresis was performed at 100 V for 3 h. Gels were then washed twice in zymography washing buffer (2.5 % Triton X-100 in double-distilled H_2O) at room temperature to remove SDS, followed by incubation at 37 °C for 12–16 h in zymography reaction buffer [40 mM Tris–HCl (pH 8.0), 10 mM $CaCl_2$, and 0.02 % NaN_3], stained with Coomassie blue R-250 (0.125 % Coomassie blue R-250, 0.1 % amino black, 50 % methanol, and 10 % acetic acid) for 1 h and destained with destaining solution (20 % methanol, 10 % acetic acid, and 70 % double-distilled H_2O). Non-stained bands representing levels of the latent form of MMP-9 were quantified by densitometrically using a digital imaging analysis system. Visualization of uPA activity was performed by casein plasminogen zymography, as described previously (Tung et al. 2013).

Immunofluorescence staining

The expression of MMP-9, uPA, and NF-κB were assayed by immunofluorescence staining, as described previously (Chen et al. 2013c). After the indicated metformin treatment, these cells were placed onto an 8-well Lab-Tek chambered cover glass (Thermo, Rochester, NY), washed with PBS, and fixed in 4 % paraformaldehyde for 20 min. Cells were permeabilized with PBS containing 0.25 % Triton X-100 for 30 min and blocked with 2 % bovine serum albumin for 20 min. Cells were then incubated with a 1:200 dilution of uPA, MMP-9 and NF-κB antibodies in PBS buffer containing 10 % bovine serum albumin overnight at 4 °C, and washed and incubated with a 1:200 dilution of TRITC-conjugated anti-mouse IgG. Cells were examined and photographed by immunofluorescence microscopy.

Western blotting

Protein expression was evaluated using Western blot analysis, as previously described (Yiang et al. 2009). Supernatant samples containing 30 μg of total protein were resolved by 10 or 12.5 % SDS-PAGE depending on the size of the target proteins, and blotted onto a polyvinylidene fluoride membrane (Millipore, Belford, MA). After blocking, the

membrane was incubated with anti-MMP-9, anti-uPA, anti-ERK1/2, anti-phosphorylated ERK1/2, anti-p38, anti-phosphorylated p38, anti-JNK1/2, anti-phosphorylated JNK1/2, anti-NF-κB (p65), anti-Lamin B, anti-α-tubulin, and anti-β-actin. Blots were then incubated with HRP-conjugated anti-mouse, anti-goat, or anti-rabbit antibodies at room temperature for 2 h. Signals were detected via enhanced chemiluminescence using the Immobilon Western-HRP Substrate (Millipore, Billerica, USA).

RNA extraction and quantification reverse transcriptase-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from HCC cells using Trizol reagent (Invitrogen Life Technologies), according to the manufacturer's instructions (Yu et al. 2012). cDNA was amplified from 2 μg of total RNA in a final volume of 20 μL using ImProm-II™ reverse transcriptase at 42 °C for 65 min and 99 °C for 5 min before chilling on ice for 10 min. The PCR reaction was performed SYBR Green Master Mix reagent. RT-PCR primer sequences were: MMP-9 (forward, 5′-CCTGCCAGTTTCC ATTCATC-3′; reverse, 5′-GCCATTCACGTCGTCCTTA T-3′); uPA (forward, 5′-TTGCGGCCATCTACAGGAG-3′; reverse, 5′-ACTGGGGATCGTTATACATC-3′); and β-actin (forward, 5′-GCACTCTTCCAGCCTTCCTTCC-3′; reverse, 5′-TCACCTTCACCGTTCCAGTTTTT-3′). Relative gene expression was obtained after normalization with endogenous β-actin and determination of the difference in threshold cycle (Ct) between treated and untreated cells using $2^{-\Delta\Delta Ct}$ method. PCR was performed on an ABI StepOneTM Real-Time PCR System (Applied Biosystems).

Preparation of cell lysate and nucleus fraction

Cell lysates were prepared by lysing cells in RIPA buffer (50 mM Tris at pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.25 % Na-deoxycholate, 1 % NP-40, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and 1 μg/ml aprotinin) via sonication. Soluble extracts were collected from the supernatant after centrifugation at 15,000*g* for 10 min. Nucleus purification was performed as described previously with a slight modification (Tsai et al. 2014). Cells were lysed in buffer A (20 mM HEPES at pH 7, 10 mM KCl, 2 mM $MgCl_2$, 0.5 % NP-40, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and 1 μg/ml aprotinin) on ice, ground in a glass dounce homogenizer, and centrifuged at 1,500*g* for 10 min. The supernatant was the cytosolic fraction, and the nuclear pellet was isolated and washed. The nuclei were lysed in NETN buffer (20 mM Tris at pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5 % NP-40, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and 1 μg/ml aprotinin) via sonication and centrifuged at 12,000*g* for 20 min. The nuclear fraction was collected.

Luciferase assay

The luciferase assay was performed, as described previously (Chou et al. 2013). SK-Hep-1 cells were grown in a 24-well dish and transiently co-transfected with 0.5 μg of pGL3, pGL3-MMP-9, or pGL3-uPA, and 0.25 μg of cytomegalovirus (CMV)-β-galactosidase plasmids using Jet-PEI reagent. Cells were then lysed and luciferase activity was measured using a luminometer (Luminoscan Ascent, Thermo Electron Co.). Luciferase activity was normalized to β-galactosidase activity in all cell lysates using an assay system (Promega, Madison, WI), and expressed as an average of three independent experiments.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as described previously (Tsai et al. 2012). The sequences of the primers specific to the promoter of uPA gene are 5′-AGCATGACAGCCTC-CAGCCAAGTA-3′ (forward), and 5′-ACGTGACCA-GAACATAAACAGAGA-3′ (reverse), and the promoter of MMP-9 gene are 5′-GAGGCTGCTACTGTCCCCT-3′ (forward), and 5′-GCTAGGCAAGGCTGGGGA-3′ (reverse). PCR products were analyzed on 2 % agarose gels and images were analyzed with NIH ImageJ densitometric measurements.

Statistical analysis

Data were expressed as mean ± SEM of three independent experiments and analyzed using Instat software (GraphPad Prism4, San Diego, CA). The Student's *t* test or one-way analysis of variance (ANOVA) followed by the post hoc Tukey's multiple comparison method were used to determined statistical differences between parametric data. $P < 0.05$ and $P < 0.01$ were considered statistically significant.

Results

Metformin decreased cell migration and invasion in three HCC cells

Using an MTT assay, we examined the effects of metformin on normal hepatic cells (THLE-2) and three types of HCC cells (SK-Hep-1, HA22T/VGH and Huh-7) on cell viability after incubating them for 24 h at various concentrations of metformin (0, 10, 30, and 50 mM). Metformin at concentrations lower than 50 mM had no cytotoxic effects on HCC cells, whereas metformin at a concentration of 70 mM decreased cell viability by about 35–55 % (Fig. 1a–c). In the THLE-2 cells, no significant cytotoxicity was detected (Fig. 1d). Accordingly, all cells were incubated

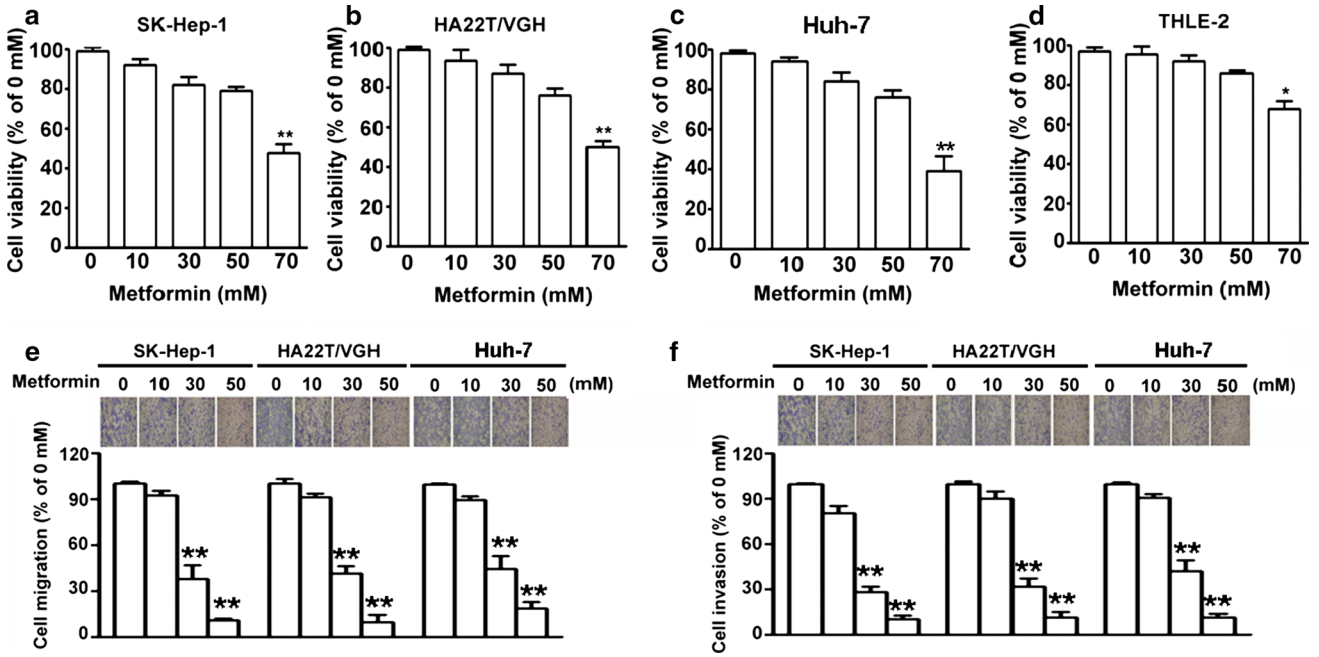

Fig. 1 Effects of metformin on the cell viability, migration, and invasion of HCC cells. **a**–**d** Cell viability of human HCC cells (SK-Hep-1, HA22T/VGH and Huh-7) and normal hepatic cells (THLE-2) after exposure to metformin. Cells were exposed to various concentrations of the metformin (0, 10, 30, and 50 mM) for 24 h, and assessed using an MTT assay. The results were expressed as percentages of cell viability. **e**, **f** The migration and invasion of SK-Hep-1, HA22T/VGH and Huh-7 cells were determined by using a migration assay and Matrigel-invasion assay. Cells in the lower surface of the Borden chamber were stained and photographed under a light microscope at ×400 magnification. Data were represented as mean ± SE of three independent experiments performed in triplicate. $^{**}P < 0.01$ versus untreated control (0 mM)

for 24 h at concentrations of 10, 30, and 50 mM of metformin in all subsequent experiments. One important characteristic of metastasis is the migratory and invasive ability of tumor cells. After treating SK-Hep-1, HA22T/VGH and Huh-7 cells with the above-mentioned concentrations of metformin for 24 h, metformin inhibited the migration (Fig. 1e) and invasion (Fig. 1f) of HA22T/VGH, SK-Hep-1 and Huh-7 cells in a concentration-dependent manner. These findings suggest that metformin significantly inhibits the migration and invasion of highly invasive HCC cells.

Metformin inhibits MMP-9 and uPA activity, expression, and mRNA levels in HCC cells

MMP-9 and uPA are thought to play a critical role in HCC cancer cell migration and invasion by stimulating the degradation of the ECM, and their increased expression is associated with disease progression (Qin and Tang 2002). SK-Hep-1 and HA22T/VGH cells displayed significant reductions in MMP-9 and uPA protein expression and activity levels after being treated with metformin in a concentration-dependent manner, as determined by Western blotting (Fig. 2a), and gelatin and casein zymography (Fig. 2b). Using immunofluorescence assay, it was revealed that metformin also inhibited MMP-9 and uPA activity in SK-Hep-1 and HA22T/VGH cells (Fig. 2c). To explore whether metformin regulates MMP-9 and uPA at the transcriptional level, quantitative RT-PCR analysis was performed and revealed that metformin had inhibitory effects on the mRNA levels of MMP-9 and uPA in both HCC cells (Fig. 2d). These results indicate that metformin suppresses the migration and invasion ability of HCC cells via a down-regulation of MMP-9 and uPA expression and activity.

Metformin inhibits the phosphorylation of ERK1/2 and JNK1/2 in HCC cells

To examine the effects of metformin on the expressions of MAPK and Akt pathways were investigated by Western blots to clarify the underlying mechanisms. We showed that metformin dose-dependently inhibited the phosphorylation of ERK1/2 and JNK1/2 (Fig. 3a, c). However, metformin had no significant effects on the phosphorylation and total protein levels of p38 and Akt (Fig. 3b, d). To further investigate whether the inhibition of MMP-9 and uPA by metformin was mainly occurring through an inhibition of the ERK1/2 and JNK1/2 signaling pathway, ERK an inhibitor (PD98059) and JNK1/2 inhibitor (SP600126) were used. After being exposed solely to metformin, SP600125, or PD98059, there was a significant decrease in the protein

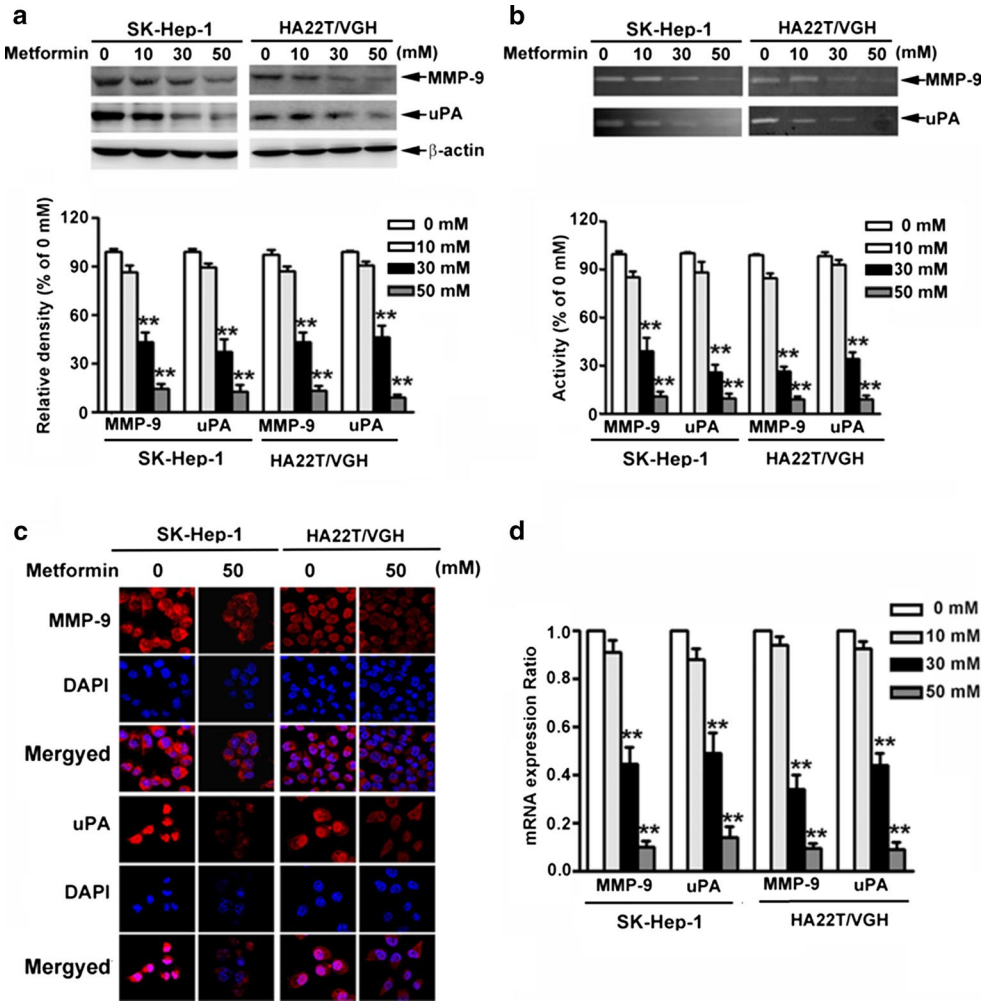

Fig. 2 Effects of metformin on MMP-9 and uPA expression levels in HCC cells. **a** SK-Hep-1 and HA22T/VGH cells were treated with various concentrations of metformin (0, 10, 30, and 50 mM) for 24 h. Protein levels of MMP-9 and uPA from treated cell lysates were analyzed by Western blotting. β-actin was used as a loading control. **b** Conditioned media were collected, and MMP-9 and uPA activities were determined by gelatin or casein–plasminogen zymography. MMP-9 and uPA activities were quantified by densitometric analysis. **c** Cells were fixed and immunostained with an anti-MMP-9 or anti-uPA antibody (*red*), and cell nuclei were counterstained with DAPI reagent. **d** Total RNA was isolated and qRT-PCR analysis was performed to measure the mRNA expression levels of MMP-9 and uPA. Data are presented as the mean ± SE of at least three independent experiments. $^{**}P < 0.01$, compared with that of the untreated control (0 mM) (color figure online)

expression and activity of MMP-9 and uPA. After metformin treatment with either PD98059 or SP600125, protein expression and activities of MMP-9 and uPA were further reduced compared to treatment with any single substance (Fig. 4a–c). As shown in Fig. 4d, e, pretreatment with metformin, SP600125, or PD98059 significantly inhibited the migration and invasion of human HCC cells, respectively. Additionally, metformin combined with either PD98059 or SP600125 induced a greater inhibition of migration and invasion of human HCC cells. These results indicate that metformin inhibits the migration and invasion of HCC cells through the ERK1/2 and JNK1/2 pathway.

Metformin inhibits the transcriptional activity of MMP-9 and uPA by disrupting nuclear translocation and activity of NF-κB

To investigate the effects of metformin in modulating MMP-9 and uPA transcriptional activity, transient transfections were performed using human MMP-9 and uPA luciferase promoter constructs. Treatment with metformin led to a decrease in MMP-9 and uPA promoter activity in a dose-dependent manner (Fig. 5a). It is known that the activation of transcription factors, AP-1 and NF-κB, is pertinent to malignant tumor progression and alters gene expression patterns, leading to cancer metastasis (Huang and Hung 2013). The effects of metformin on the nuclear translocation of NF-κB, c-jun, and c-fos in SK-Hep-1 cells was investigated by Western blotting. Metformin significantly inhibited NF-κB (p65), but not c-jun or c-fos, in the nuclear extracts of SK-Hep-1 cells in a dose-dependent manner (Fig. 5b). Additionally, the binding capability of NF-κB (p65) on the promoters of MMP-9 and uPA genes was repressed after treatment with metformin at 30 and 50 mM in SK-Hep-1 cells (Fig. 5c). We explored whether activation of ERK1/2 and JNK1/2 leads to MMP-9 and uPA transcription via activation of NF-κB (p65). Pretreatment of cells with PD98059 or SP600125 significantly inhibited metformin-induced NF-κB translocation to the nucleus (Fig. 5d, e). Additionally, ChIP analysis showed that when metformin is combined with PD98059 or SP600125 in SK-Hep-1 cells, NF-κB (p65) binding to MMP-9 and uPA

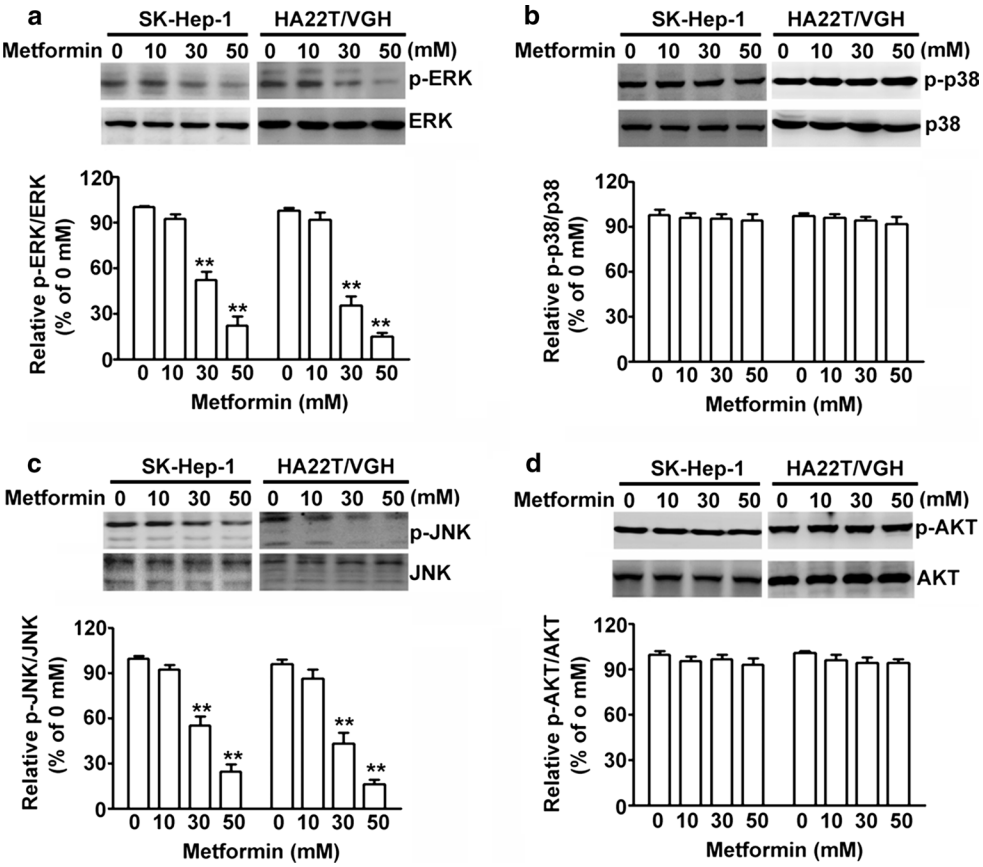

Fig. 3 Effects of metformin on the MAPK and Akt pathway in HA22T/VGH and SK-Hep-1 cells. Cells were treated with various concentrations of metformin (10, 30, and 50 mM) for 24 h. Western blot analysis was used to examine total expression and phosphorylation levels of **a** ERK1/2, **b** p38 MAPK, **c** JNK1/2, and **d** Akt. β-actin was used as an internal control for protein equal loading. Data are presented as the mean ± SE of three separate experiments. $^{**}P < 0.01$ compared with untreated control (0 mM)

promoter regions was significantly decreased (Fig. 5f). These results suggest that metformin inhibits the nuclear translocation of NF-κB through the inhibition of the ERK1/2 and JNK1/2 signaling pathway and reduces its binding to the promoters of MMP-9 and uPA, thereby mediating the inhibition of MMP-9 and uPA expression in human highly invasive HCC cells.

Combination of metformin and sorafenib inhibits HCC cell invasion through inhibition of ERK1/2 and JNK1/2 activation

Recently, studies have supported the use of sorafenib as a first-line systemic therapy for advanced HCC. Therefore, we analyzed the effects of metformin combined with sorafenib for treating SK-Hep-1 and HA22T/VGH cells. Using an MTT assay, metformin co-treatment with different concentrations of sorafenib (1 and 2 μM) did not increase the cytotoxic effects in SK-Hep-1 and HA22T/VGH cells compared with treatment with sorafenib or metformin alone (Fig. 6a). Next, we investigated whether metformin enhances the chemosensitivity of highly invasive HCC cells to sorafenib. HCC cells were treated with various doses of either sorafenib alone or in combination with metformin by migration and invasion was assessed. After being exposed solely to metformin or sorafenib (1 and 2 μM), there was a significant decrease in migration and invasion. Combination treatment (metformin with 1 or 2 μM sorafenib) for 24 h significantly enhanced the suppressing effects of sorafenib on the migration and invasion of SK-Hep-1 (Fig. 6b) and HA22T/VGH (Fig. 6c) cells. These data indicate that metformin and sorafenib had synergistic anti-metastatic effects on HCC cells.

Combination treatment inhibited nuclear translocation and binding activities of NF-κB dependent on MMP-9 and uPA expression

To test whether the combination of metformin and sorafenib inhibits cell invasion through downregulation of the ERK1/2 and JNK1/2 pathway. Combination treatment with metformin and sorafenib (1 and 2 μM) for 24 h enhanced the suppressing effect of sorafenib on the phosphorylation of ERK1/2 and JNK1/2 (Fig. 7a), protein expression (Fig. 7a) and activities of MMP-9 and uPA (Fig. 7b). We further sought to validate that the combination of metformin and sorafenib suppresses NF-κB nuclear translocation and its binding to the MMP-9 and uPA promoters. It was found that treatment with either metformin or sorafenib resulted in a decrease of NF-κB nuclear

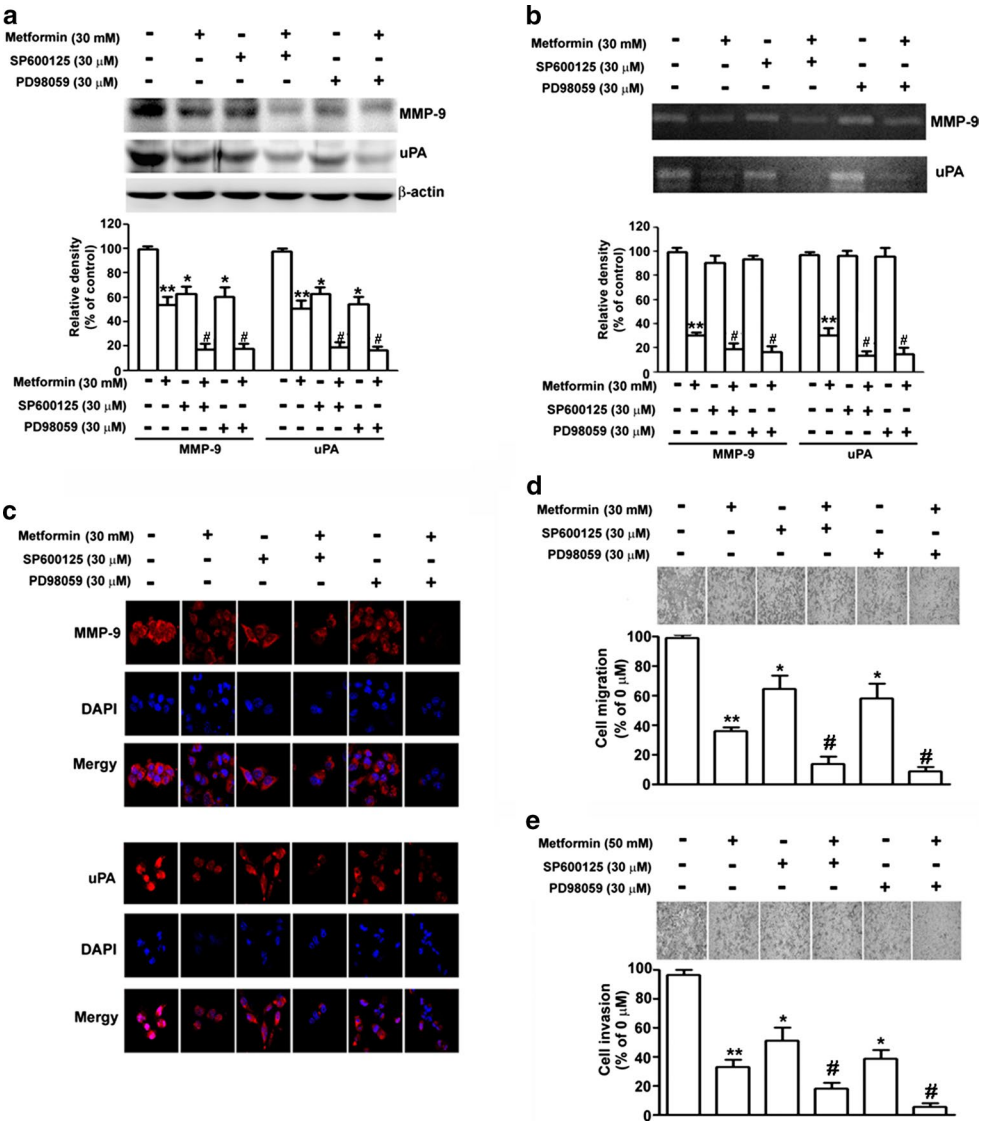

Fig. 4 Effects of PD98059 or SP600125 on the metformin-induced inhibition of cell migration and invasion. **a** SK-Hep-1 cells were treated with or without metformin and/or PD98059 and SP600125, respectively, and then, cells were subjected to Western blotting analysis; **b** gelatin/casein zymography; and **c** immunofluorescence staining. β-actin was used as an internal control for protein equal loading. The migration (**d**) and invasion (**e**) of SK-Hep-1 cells after treatment were determined. Data were represented as mean ± SE of three independent experiments performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, control versus metformin; $^{\#}P < 0.01$, metformin versus PD98059 or SP600125 inhibitor plus metformin

translocation (Fig. 7c) and its binding to the MMP-9 and uPA promoter (Fig. 7d). Furthermore, these suppressive effects were further enhanced after a combination treatment of metformin and sorafenib (Fig. 7d). Based on these findings, we propose a model for the molecular mechanisms through which metformin inhibits the migration and invasion of HCC cells, as well as enhances their chemosensitivity to sorafenib (Fig. 8).

Discussion

Despite new advances in our understanding of the molecular mechanisms of HCC cells and the development of new treatment strategies, HCC still remains one of the leading causes of cancer death worldwide, including in sub-Saharan Africa and eastern Asia (Reuben 2003). Novel approaches are, therefore, urgently needed for the treatment of this disease and prevention of its metastasis. In recent years, many reports found that metformin may have anti-tumor properties, which has been verified in treating some alimentary and reproductive system tumors. However, the anti-metastatic effects of metformin in HCC cells remain unknown. In this study, our results indicate for the first time that: (1) metformin is a potent inhibitor of cell migration and invasion of highly invasive HCC cells; (2) metformin inhibits the expression and activities of MMP-9 and uPA in HCC cells; (3) metformin inhibits the activities of ERK1/2 and JNK1/2 in HCC cells; (4) metformin suppresses MMP9 and uPA via inhibition of the JNK1/2 and ERK1/2 signaling pathway, exerts inhibitory effects on the nuclear translocation of the transcription factor NF-κB and its binding to MMP9 and uPA promoters, reduces levels of MMP9 and uPA, and has anti-metastatic effects; and (5) metformin

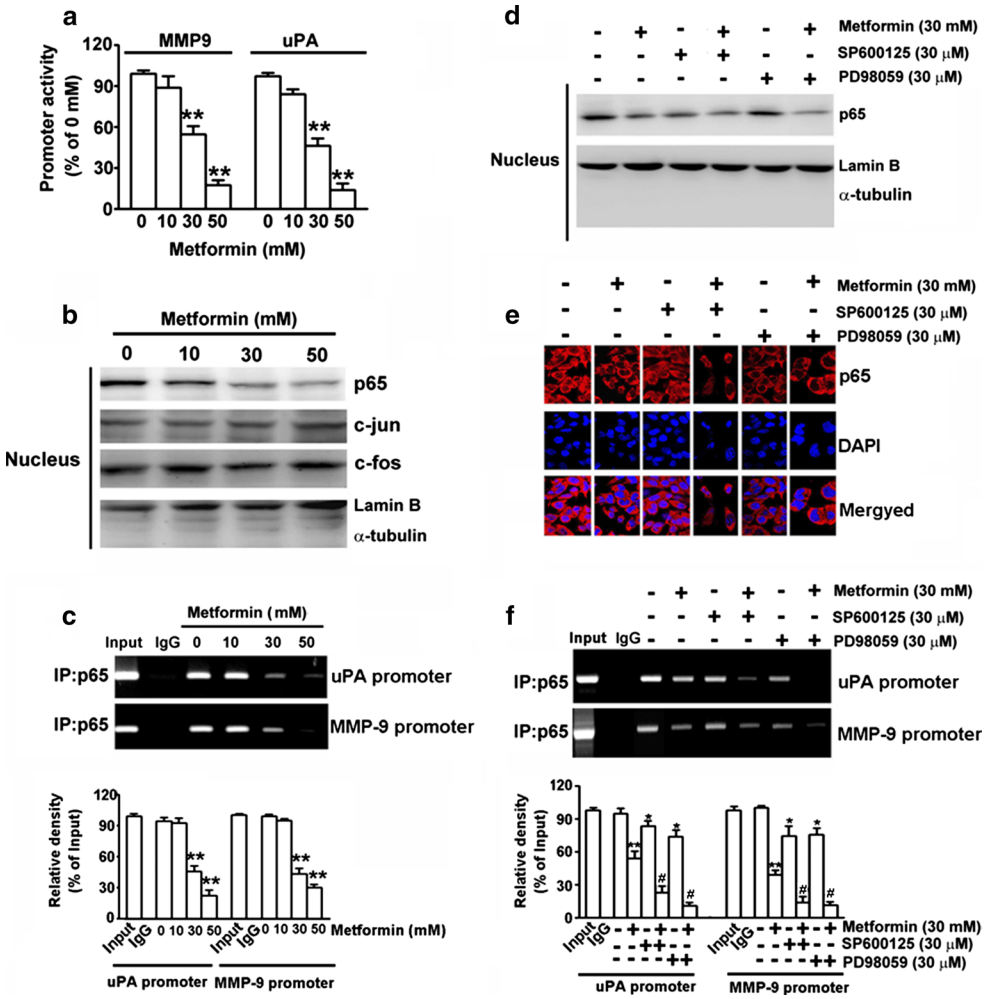

Fig. 5 The ERK1/2 and JNK1/2 signaling pathway is involved in the metformin-induced inhibition of localization and DNA binding activity of NF-κB on the MMP-9 and uPA promoter regions in SK-Hep-1 cells. **a** Cells transfected with luciferase reporter plasmids containing the promoter region of either MMP-9 or uPA were treated with increased concentrations of metformin for 24 h. Cell lysates were extracted following treatment, and the activity of MMP-9 or uPA promoters was determined by a luciferase activity assay. The plot demonstrates the relative activity of the MMP-9 or uPA promoters from three independent experiments. **b** Cells were treated with various concentration of metformin (0, 10, 30, and 50 mM) for 24 h, and the effects of metformin on the nuclear protein levels of NF-κB (p65), c-Jun, and c-Fos were examined by Western blotting. Lamin B and α-tubulin were used as markers of nuclear and cytosolic fractions, respectively. **c** After metformin treatment, protein and chromatin within cells were cross-linked, and the DNA binding ability of NF-κB (p65) on the MMP-9 and uPA gene promoters was determined by using a ChIP assay. The *bottom plot* shows the relative quantitative results compared to that of the input. **d**, **e** Nuclear protein levels and localization of NF-κB (p65) and its binding activity on the MMP-9 and uPA gene promoters (**f**) after treatment with or without metformin and/or PD98059 and SP600125, respectively. The *bottom plot* shows the relative quantitative results compared to that of the input. Data are presented as the mean ± SE from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, control versus metformin; $^{\#}P < 0.01$, metformin versus PD98059 or SP600125 inhibitor plus metformin

has the potential to enhance the anti-metastatic effect of sorafenib by inhibiting the expression of MMP9 and uPA of highly invasive HCC cells.

HCC is one of the most major causes of malignancy-induced deaths, and its poor prognosis is mostly due to its local invasion and subsequent metastasis, which is the most critical parameter for determining survival in HCC patients (Morgan et al. 2004). For distant metastases to occur, malignant cells must have the ability to migrate and invade, destroy intercellular relationships, lyse the ECM, and increase the adhesion between cells and the ECM (Deryugina et al. 1998; Webb and Vande Woude 2000). Among these steps, MMPs and uPA play a key role in degrading the ECM and allowing metastatic cells to have access to the vasculature, invade, and migrate into the target organ, thereby resulting in tumor metastasis. (Liotta and Stetler-Stevenson 1991). uPA expression can be modulated by various intracellular upstream signaling cascades, particularly the MAPK pathway (Blasi and Sidenius 2010). Studies have shown that MAPK, which is composed of at least

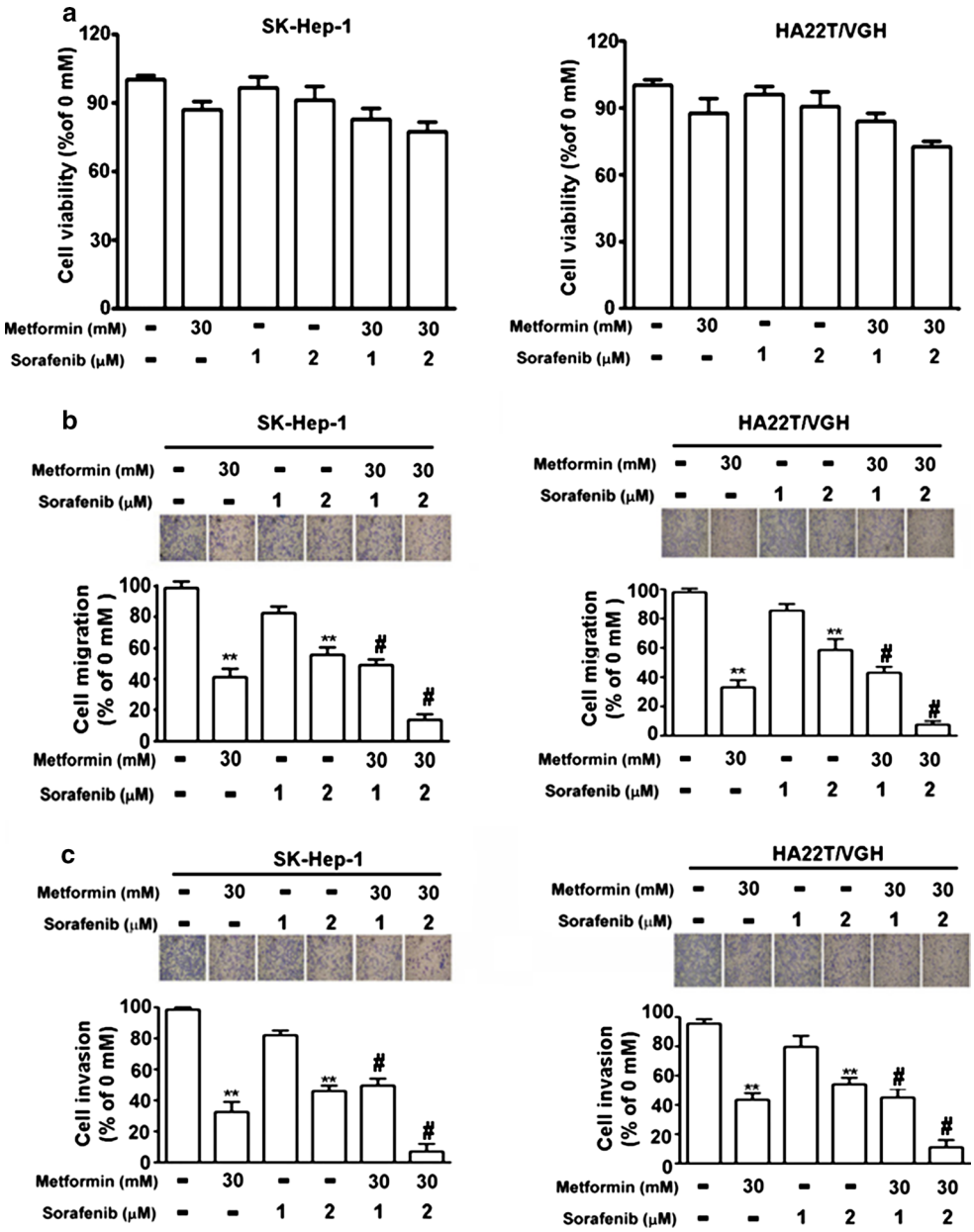

Fig. 6 Effects of metformin alone or in combination with sorafenib on cell viability, migration, and invasion of human HCC cells. **a** Cells were incubated with 30 μM of metformin in combination with or without sorafenib (1 or 2 μM) for 24 h, after which cell viability was measured using the MTT assay. The migration and invasion of SK-Hep-1 and HA22T/VGH cells was determined by using **b** a migration assay and **c** a Matrigel-invasion assay. Data were presented as mean ± SE of three independent experiments performed in triplicate. $^{**}P < 0.01$, control versus alone metformin or sorafenib (1 or 2 μM); $^{\#}P < 0.01$, metformin versus sorafenib (1 or 2 μM) plus metformin

four subfamilies, namely ERK1/2, JNK, p38, and ERK5, can integrate extracellular stimulations into various cellular activities, such as cell proliferation, survival, or migration (Blasi and Sidenius 2010). Elevations in JNK phosphorylation were found to be correlated with the pathogenesis of HBV-associated HCC (Guo et al. 2005), and increased JNK phosphorylation was found in 23 out of 41 HCC samples by microarray (Hui et al. 2008) and overactivated in more than 50 % of Chinese HCC patients (Chang et al. 2009). In human HCC cells, an upregulation of the MAPK pathway results in invasion and regulates the expression levels of MMPs and uPA. (Lu et al. 2012; Zhu et al. 2012). Hence, interrupting MMPs and uPA expression may be an approach used in anti-metastatic therapy, including human HCC cells. Consistent with this hypothesis, in this study, the expression levels of uPA and MMP-9 of HCC cells were found to be decreased after being treated with metformin.

Metformin is generally considered non-toxic with a wide therapeutic window, no associated hypoglycemia, and only rare reports of lactic acidosis. Recently, several studies have demonstrated that metformin has effects against several cancer types, including breast cancer (Phoenix et al. 2009), pancreatic cancer (Li et al. 2009), prostate cancer (Ben Sahra et al. 2008), gliomas (Isakovic et al. 2007), and HCC (Chen et al. 2013a). There have been studies demonstrating promising results in inhibiting HCC cells via the suppression of the MAPK signaling pathway. In human HCC cell

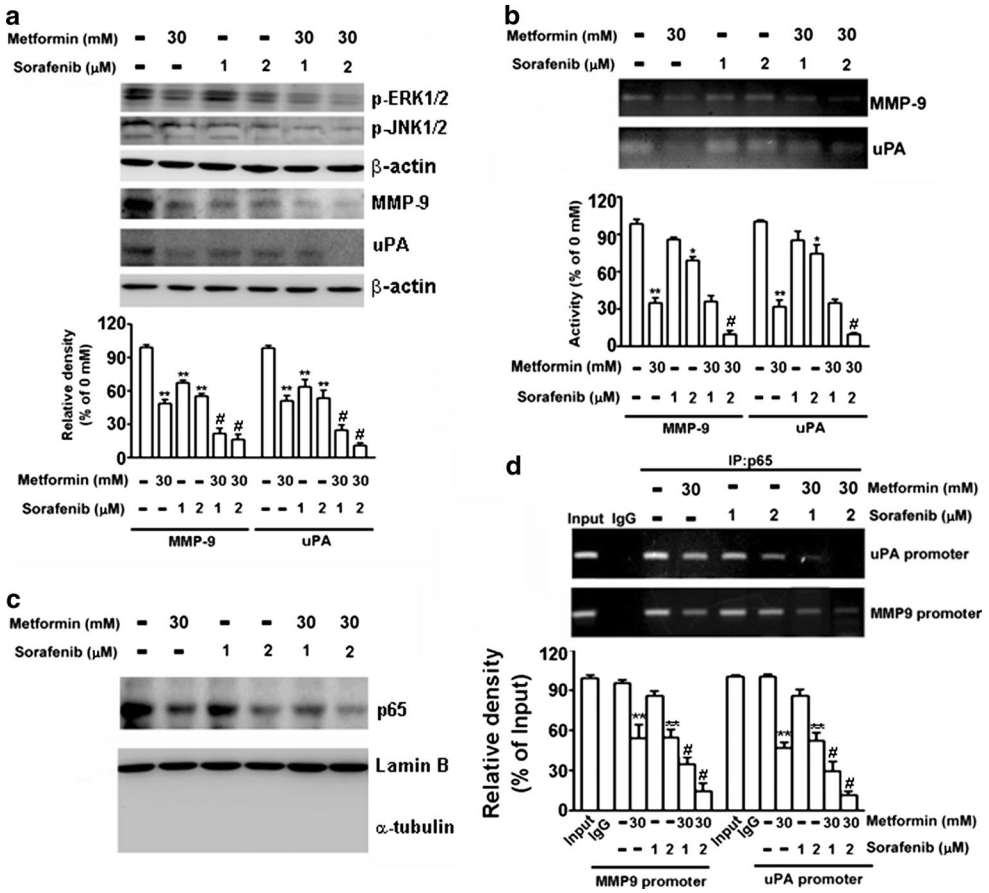

Fig. 7 Effects of MMP-9 and uPA expression on metformin alone or in combination with sorafenib in SK-Hep-1 cells. **a** Cells were incubated with 30 μM of metformin in combination with or without sorafenib (1 or 2 μM) for 24 h, and the effects of metformin alone or in combination with sorafenib (1 or 2 μM) on phosphorylation of ERK1/2, JNK1/2, MMP-9 and uPA expression were examined by Western blotting. β-actin was used as loading control. **b** Conditioned media were collected, and MMP-9 and uPA activities were determined by gelatin or casein–plasminogen zymography. MMP-9 and uPA activities were quantified by densitometric analysis (**c**). The effects of metformin alone or in combination with sorafenib (1 or 2 μM) on the nuclear protein levels of NF-κB (p65) were examined by Western blotting. Lamin B and α-tubulin were used as markers of nuclear and cytosolic fractions, respectively. **d** The binding to the MMP-9 and uPA gene promoters after incubation with 30 μM of metformin with or without sorafenib (1 or 2 μM) by ChIP assay, respectively. The *bottom plot* shows the relative quantitative results, compared to that of the input. Data were presented as mean ± SE of three independent experiments performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, control versus alone metformin or sorafenib (1 or 2 μM); $^{\#}P < 0.01$, metformin versus sorafenib (1 or 2 μM) plus metformin

lines, treatment with Genistein was found to modulate their invasive and metastatic potential by suppressing MMP-9 transcription by inhibiting AP-1 and NF-κB activity through a suppression of ERK and JNK phosphorylation, as well as NF-κB nuclear translocation via inhibition of the IκB signaling pathway (Wang et al. 2014). By treating HepG2 cells with melatonin, MMP-9 activity was reduced, and cell invasion and motility were inhibited through a downregulation of MMP-9 gene expression resulting from a suppression of the NF-κB signaling pathway and upregulation of the MMP-9-specific inhibitor (Ordonez et al. 2014). Moreover, after treating HCC cells (MHCC97H) with baicalein, the expression levels of MMP-9 and uPA, and ERK1/2 phosphorylation were decreased, indicating that baicalein inhibits tumor cell invasion and metastasis by reducing cell motility and migration via suppression of the ERK pathway (Chen et al. 2013b). Consistent with these studies, we found that the expression levels of uPA and MMP-9 in HCC cells were decreased via an inhibition of the ERK1/2- and JNK1/2-mediated singling pathway after being treated with metformin, an ERK inhibitor, and a JNK inhibitor. Moreover, we found that metformin inhibits the expression levels of nuclear NF-κB, as well as the binding of NF-κB to the MMP-9 and uPA promoter, thereby suppressing the expression of MMP-9 and uPA to inhibit the migration and invasion of HCC cells. These results suggest that metformin may be used as a potential anti-metastatic therapy for HCC.

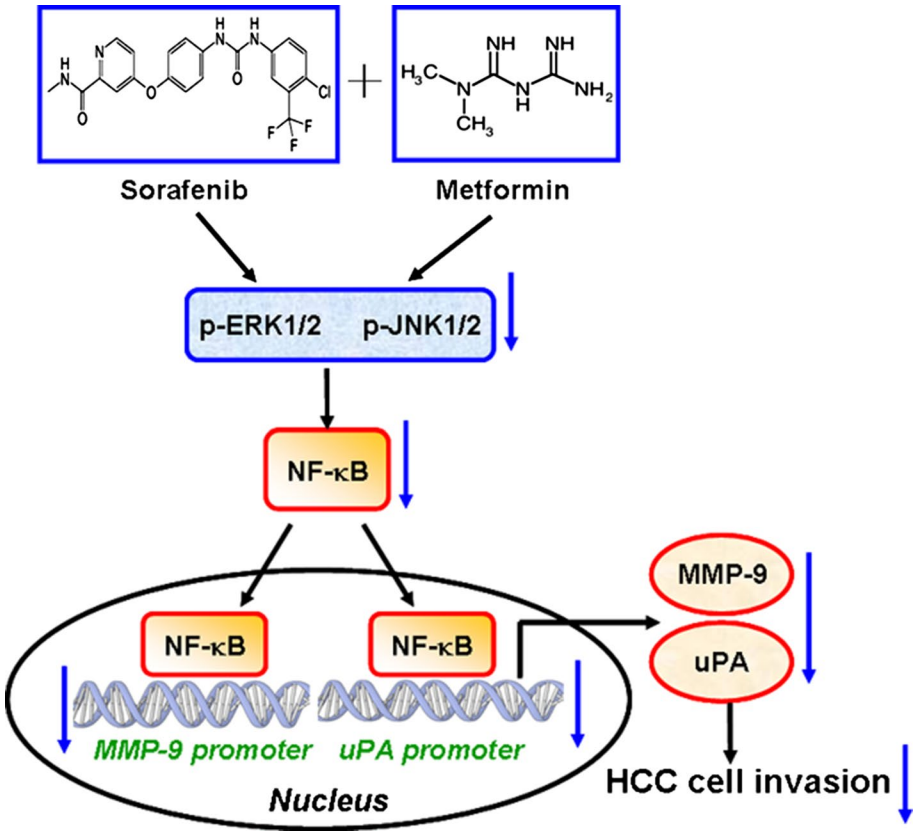

Fig. 8 A proposed model for the mechanism of action of metformin in combination with sorafenib in suppressing HCC cell migration and invasion through an inhibition of MMP-9 and uPA expression via the MAPK and NF-κB signaling pathway

Sorafenib, an oral multi-tyrosine kinase inhibitor, was the first and remains the only drug to demonstrate survival benefits in patients diagnosed with advanced HCC (Huang et al. 2013). In the SHARP and Asia-Pacific trials, sorafenib prolonged median survival and time-to-progression in advanced HCC, but all of the endpoints did not reach ideal levels (Llovet et al. 2008). Given that there is no single dominant pathway responsible for hepatocarcinogenesis, a single-targeted agent will not achieve sustained complete responses in HCC. However, it is conceivable to either inhibit signaling at different levels in one of the main pathways or to inhibit two or three different pathways at the same time. Recently, sorafenib has been combined with radiotherapy (median survival times in the treated and control groups were 15.7 and 7.8 months, respectively) (Theysohn et al. 2012) or doxorubicin (disease control rates in treated and control groups were 69 and 40 %, respectively) (Richly et al. 2009) demonstrating promising results. The combination of metformin with anti-cancer drugs has been previously suggested as a potential strategy for cancer therapy (Teixeira et al. 2013). In human SK-Hep-1 cells, the combination of sorafenib with β-ionone showed synergistic inhibition of cell invasion and migration via an inhibition of MMP-2 and MMP-9 activities, and phosphorylation of focal adhesion kinase and Rac1 proteins (Huang et al. 2012). Conversely, using metformin in DM patients with HCC undergoing radiofrequency ablation therapy induces a favorable overall survival compared to patients without metformin therapy (Chen et al. 2011). In vitro studies demonstrated that treating HCC cells with metformin plus ionizing radiation resulted in a significant decrease in cell viability, and blocked the cell cycle in the G2/M phase by initiating stress and decreasing DNA repair (Liu et al. 2012). Moreover, combining metformin with the PIAF regimen (cisplatin/interferon α-2b/doxorubicin/5-fluorouracil) increased oxidative stress within the malignant liver stem-like cells (Nangia-Makker et al. 2014; Petrushev et al. 2012). After transplanting Huh7 cells into NOD/SCID and treating them with metformin and/or sorafenib, combination therapy appeared more effective at suppressing the growth of subcutaneous tumors than monotherapy (Saito et al. 2013). Similarly, we also found that co-administrating HCC cells with metformin and sorafenib induced a greater inhibition of MMP-9 and uPA expression and activity, as well as cell migration and invasion.

To the best of our knowledge, this is the first study to demonstrate that metformin inhibits HCC cell migration and invasion by decreasing uPA and MMP-9 expression and activity. Metformin-induced inhibition of uPA and MMP-9 was found to be due to its ability to suppress the activation of the ERK1/2 and JNK1/2 signaling pathway, as well as NF-κB nuclear translocation, and subsequently,

NF-κB transcriptional activation. Moreover, metformin may enhance the ability of sorafenib in inhibiting HCC cells migration and invasion via a suppression of MMP-9 and uPA expression (Fig. 8). These findings suggest that metformin may be used as a promising anti-metastatic therapy and may improve outcomes of conventional chemotherapy used in HCC.

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Conflict of interest The authors declare that there is no conflict of interest.

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