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Lucidenic acid inhibits PMA-induced invasion of human hepatoma cells through inactivating MAPK/ERK signal transduction pathway and reducing binding activities of NF-κB and AP-1

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Ganoderma lucidum **has been reported to be associated with suppressed motility, invasion and metastasis of several types of cancers, but its mechanism of action remains unclear. In our previous study, lucidenic acids A, B, C and N were isolated from a new strain of *G.lucidum* and all of them were found to have potential anti-invasive activity on phorbol-12-myristate-13-acetate (PMA)-induced $HepG_2$ cells by suppressing the matrix metalloproteinase (MMP)-9 activity. Here, the lucidenic acid B (LAB) was used to explore its mechanisms underlying MMP-9 expression of $HepG_2$ cells. The results showed that the LAB suppressed PMA-induced MMP-9 activity in a dose-dependent transcriptional level. The suppression of PMA-induced MMP-9 expression of $HepG_2$ cells by LAB was through inactivating phosphorylation of extracellular signal-regulated kinase (ERK) 1/2. The treatment of mitogen-activated protein kinase kinase (MEK) inhibitors (PD98059 and U0126) and LAB to $HepG_2$ cells could result in a synergistic reduction on the MMP-9 expression along with an inhibition on cell invasion. Moreover, LAB also strongly inhibited PMA-stimulated nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) DNA-binding activities of $HepG_2$ cells in dose-dependent manners. A dose-dependent inhibition on protein levels of NF-κB, c-Jun and c-Fos in nuclear by LAB treatment was further observed. In conclusion, we demonstrated that the anti-invasive effects of the LAB on the PMA-induced $HepG_2$ cells might be through inhibiting the phosphorylation of ERK1/2 and reducing AP-1 and NF-κB DNA-binding activities, leading to downregulation of MMP-9 expression.**

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the Southeast Asia, including Taiwan. HCC is a hypervascular tumor, in which venous invasion is common and often progresses to give rise to intrahepatic and extrahepatic metastasis (1). The invasion and metastasis of cancer cells are known to be the primary causes of human cancer death and are complicated processes involving a group of proteolytic enzymes, which participate in the degradation of environmental barriers such as extracellular matrix and basement membrane. Among these enzymes, the matrix metalloproteinases (MMPs), which are a family of zinc-dependent endopeptidases, are deeply involved in the invasion and metastasis of various tumor cells (2,3). The expressions of *MMP* gene in various tumor cells were known to regulate at a transcriptional, posttranscriptional or (and) protein levels. Therefore, MMP protein expressions and their regulatory pathways were considered as promising targets for anticancer drugs and chemopreventive agents (4). The transcriptional level of MMP expression is via various transcription factors and mitogen-activated protein kinase (MAPK) or phosphoinositide-3 kinase/protein kinase B (PI3K/Akt) pathways (5,6). The posttranscriptional level is via regulatory factors and messenger RNA (mRNA) interactions to mediate MMP mRNA stability (7). The protein level is via their activators or inhibitors and their surface localization (4,8,9). Several MMP inhibitors have been developed to downregulate the MMP expressions and used to investigate the prevention of tumor invasion and metastasis (10,11).

Type IV collagen is a major constituent of the basement membrane that separates the epithelial and stromal compartments. MMP-9 (a 92 kDa type IV collagenase or gelatinase B) and MMP-2 (a 72 kDa type IV collagenase or gelatinase A), which play critical roles in the degradation of type IV collagen, are highly expressed in various malignant tumors and closely related to the invasion and metastasis of cancer cells (12–15). The 5′ flanking region of the *MMP-9* gene contains several functional regulatory motifs that can bind with several well-characterized transcription factors, including nuclear factor-kappa B (NF-κB; −600), activator protein-1 (AP-1; −533 and −79), stimulatory protein-1 (−558) or polyoma virus enhancer activator-3 (−540) (5,16). Through one or more of these binding sites on the specific element-containing target genes, the expression of MMP-9 is regulated by various chemical or physical stimulators, including growth factors (e.g. fibroblast growth factor-2, epidermal growth factor and hepatocyte growth factor), cytokines (e.g. tumor necrosis factor-α), oncogenes (e.g. Ras) and phorbol-12-myristate-13-acetate (PMA) (17–21). Among these stimulators, PMA is a well-known selective activator of protein kinase C (PKC) (18) and can act as a tumor promoter to induce MMP-9 expression in certain cancer cells (21). MMP-9 is also regulated at a posttranslational level through an interaction with tissue inhibitor of metalloproteinase-1 (22).

Ganoderma lucidum, also called lingzhi in China, is used for preventing or treating hepatic diseases such as hepatoma, hepatopathy and chronic hepatitis (23–25). It is also used to reduce the likelihood of invasion and metastasis and to prevent occurrence or reoccurrence of various types of cancers (23,26). Dried powder and aqueous/ethanol extracts of *G.lucidum* have been used for cancer treatment in Asian countries for centuries. The anticancer and antimetastatic activities of this edible mushroom have been focused on its polysaccharide and triterpenoid components (27,28). Although triterpenoid components in *Ganoderma* species are referred to be involved in many biological effects, but literatures regarding the identification of individual bioactive triterpenoid ingredients of *G.lucidum* responsible for the claimed activities and their anti-invasive molecular mechanisms are limited. In our previous study (29), we had isolated and identified four triterpenoids, including lucidenic acids A, B, C and N, from the fruiting body of a new strain of *G.lucidum*. It was demonstrated that these four lucidenic acids could inhibit the PMA-induced invasion of HCC by suppressing MMP-9 expression, while lucidenic acid B (LAB) having the highest inhibitory effect among the four triterpenoids. In the present study, the effects of LAB on the transcriptional level of MMP expressions, the transcriptional factor NF-κB and AP-1 DNA-binding activities and the MAPK- and PI3K/Akt-signaling protein activities of $HepG_2$ cells (a highly metastatic human hepatoma cell) were investigated to explore the molecular mechanisms and signaling pathways for the involvement of lucidenic acid in hepatoma cell invasion.

Abbreviations: AP-1, activator protein-1; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; HCC, hepatocellular carcinoma; IκK, IκB kinase; JNK, c-jun N-terminal kinase; LAB, lucidenic acid B; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MMP, matrix metalloproteinase; mRNA, messenger RNA; NF-κB, nuclear factor-kappa B; PBS, phosphate-buffered saline; PI3K/Akt, phosphoinositide-3 kinase/protein kinase B; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; RT–PCR, reverse transcription–polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SPAK, stress-activated protein kinase.

Materials and methods

Materials and reagents

The LAB (7,12-dihydroxy-4,4,14-trimethyl-3,11,15-trioxo-5-chol-8-en-24-oic acid) was isolated from *G.lucidum* (YK-02) obtained from the Mycelium Center of Biotechnology R & D Institute (Shuang Hor Group, Tainan Hsien, Taiwan) and has been identified in our previous study (29). PMA was purchased from Sigma Chemical Co. (St Louis, MO). Dulbecco's modified Eagle's medium (DMEM) was purchased from HyClone (Logan, UT). Fetal bovine serum was purchased from Gibco BRL Co. (Grand Island, NY). Dimethylsulfoxide was purchased from Tedia Co. (Fairfield, OH). PD98059 (MEK inhibitor), LY294002 (PI3K inhibitor) and IκBα antibody were purchased from BioSource International (Camarillo, CA). MAPK/extracellular signal-regulated kinase (ERK) 1/2, p38 MAPK, stress-activated protein kinase (SAPK)/c-jun N-terminal kinase (JNK), protein kinase B (Akt) and NF-κB (p65), the total and phosphorylated protein antibodies were purchased from Cell Signaling Technology (Boston, MA). The β-actin monoclonal antibody was purchased from BioVision (Moutain View, CA). GF109203X (PKC inhibitor) and IκB kinase (Iκk) inhibitor peptide were purchased from CalBiochem (EMD Chemical, San Diego, CA). U0126 (MEK inhibitor) was purchased from Promega Co. (Madison, WI). Goat anti-rabbit IgG (H&L) horseradish peroxidase-conjugated antibody and goat anti-mouse IgG (H&L) horseradish peroxidase-conjugated antibody were purchased from Chemicon International (Billerica, MA).

Cell culture

Human hepatoma cells ($HepG_2$ cells) were obtained from the Bioresource Collection and Research Center (Food Industry Research and Development Institute, Hsin Chu, Taiwan). Cells were grown in DMEM, supplemented with 10% (vol/vol) fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.37% (wt/vol) $NaHCO_3$, 0.1 mM non-essential amino acid and 1 mM sodium pyruvate at 37°C, in a humidified atmosphere of 95% air and 5% CO_2. In the invasive and metastatic experiments, the cells were cultured in a serum-free medium.

Determination of MMP-2 and -9 activities by zymography

$HepG_2$ cells were incubated in serum-free DMEM in the presence of 200 nM PMA with or without lucidenic acid for a given time, and the conditioned medium was collected as samples. The samples unboiled were separated by electrophoresis on 8% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS–PAGE) containing 0.1% gelatin. After electrophoresis, the gels were washed twice in washing buffer (2.5% Triton X-100 in dH_2O) at room temperature for 30 min to remove SDS, and then incubated in reaction buffer (10 mM $CaCl_2$, 0.01% NaN_3 and 40 mM Tris–HCl, pH 8.0) at 37°C for 12 h to allow proteolysis of the gelatin substrate. Bands corresponding to the activity were visualized by negative staining using Coomassie Brilliant blue R-250 (Bio-Rad Laboratories, Richmond, CA) and molecular weights were estimated by reference to prestained SDS–PAGE markers.

Cell invasion assay

Cell invasion assay was performed according to the method of Repesh (30). $HepG_2$ cells to be tested for invasion were detached from the tissue culture plates, washed with phosphate-buffered saline (PBS) and resuspended in a serum-free DMEM (5×10^4 cells/200 μl) with the presence or absence of drugs (PMA and lucidenic acid), and then seeded onto the upper chamber of Matrigel-coated filter inserts (8 μm pore size; Becton Dickinson, Franklin Lakes, NJ). Serum-containing DMEM (500 μl) was added to the lower chamber. After 24 h of incubation, filter inserts were removed from the wells. The cells on the upper surface of the filter were wiped with a cotton swab. Filters were fixed for 10 min with methanol and stained with Giemsa dye for 1 h, and then the cells that invaded the lower surface of the filter were counted under a microscope.

Reverse transcription–polymerase chain reaction

Total RNA was prepared from $HepG_2$ cells using the 3-Zol (Trizol) reagent (MDBio, Piscataway, NJ) following the manufacturer's instructions. For reverse transcription–polymerase chain reaction (RT–PCR), 4 μg of total cellular RNA was used as template in a 20 μl reaction solution which contained 4 μl deoxyribosenucleotide triphosphate (2.5 mM), 2.5 μl oligo dT (10 pmol/μl) and RTase (200 U/μl). Then, the reaction was performed at 42°C for 1 h. The cDNA (5 μl) was amplified by PCR with the following primers: MMP-9 (480 bp), 5′-CAACATCACCTATTGGATCC-3′ (sense) and 5′-CTGTAGAGTCTCTC-GCT-3′ (antisense) and glyceraldehyde-3-phosphate dehydrogenase (307bp), 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ (sense) and 5′-AGCCTTCTCC-ATGGTTGGTGAAGAC-3′ (antisense). PCR amplification was performed under the following conditions: 25 cycles of 94°C for 1 min, 63°C for 1 min, 72°C for 2 min, followed by a final incubation at 72°C for 10 min. PCR products were analyzed by 1.8% agarose gel and visualized by ethidium bromide staining.

Preparation of cell lysates and nuclear fractions

The cell lysates and nuclear fractions were prepared using Nuclear Extraction Kit (Panomics, Redwood City, CA). Briefly, harvested cells (1×10^6 cells/6 cm plate) were washed twice with 5 ml cold 1× PBS. In total, 0.5 ml Buffer A working reagent (combining 0.5 ml 1× Buffer A, 5 μl dithiothreitol, 5 μl protease inhibitor cocktail and 20 μl 10% IGEPAL) was added to each plate, followed by transferring the plate to an ice bucket on a rocking platform at 150 r.p.m. for 10 min. A sterilized cell scraper was used to remove the cells followed by pipetting up and down several times to disrupt the cell clumps. Each sample was transferred to a 1.5 ml sterilized Eppendorf tube and centrifuged at 14 000*g* for 3 min at 4°C. The supernatant (cytosolic fraction) was removed and the pellet was kept on ice. In total, 75 μl Buffer B working reagent (combining 0.5 ml 1× Buffer B, 5 μl dithiothreitol and 5 μl protease inhibitor cocktail) was added to each pellet and vortex at the highest setting for 10 s. The Eppendorf tube was placed horizontally in an ice bucket, which was transferred to a rocking platform at 150 r.p.m. for 2 h. After centrifuged at 14 000*g* for 5 min at 4°C, the supernatant (nuclear extract) was transferred to a new Eppendorf tube for the measurement of protein concentration of each sample and was stored at −80°C.

Western blotting

Ten microgram samples of total cell lysates or nuclear fractions were size fractionated by an SDS–PAGE of 10% polyacrylamide gel and electrophoretically transferred onto a polyvinylidene fluoride membrane using Bio-Rad Mini Protean electrotransfer system (Mini-Protean, Bio-Rad Laboratories). The blot was subsequently incubated with 5% skim milk in phosphate buffered saline Tween-20 for 1 h to block non-specific binding and probed with MAPK/ERK1/2, p38 MAPK, SAPK/JNK, protein kinase B (Akt), IκBα (the total and phosphorylated proteins), β-actin and NF-κB (p65); c-Jun, c-Fos and C23 (nucleolin) were detected with each specific antibody overnight at 4°C. Then, the membrane was sequentially detected with an appropriate peroxidase-conjugated secondary antibody at room temperature for 1 h. Intensive PBS washing was performed at each interval of incubations. After the final PBS washing, signal was developed by enhanced chemiluminescence detection system and Kodak X-OMAT Blue Autoradiography Film.

Electrophoretic mobility shift assay

Binding of AP-1 and NF-κB in nuclear extracts was assessed by electrophoretic mobility shift assay (EMSA) with biotin-labeled double-stranded AP-1 (5′-TGACCCCTGAGTCAGCACTT-3′) or NF-κB (5′-CCAGTGGAATTCC-CCAG-3′) oligonucleotides and EMSA was carried out by using the EMSA Gel-Shift Kits from Panomics. Competition was performed using the unlabeled AP-1 or NF-κB oligonucleotides. Briefly, binding reaction solutions containing 10 μg of nuclear protein, 10 mM Tris, 50 mM KCl, 1 mM dithiothreitol, 5 mM $MgCl_2$, 2 μg poly (dI.dC) and 2 pmol of oligonucleotide probe were incubated for 20 min at room temperature. Specific binding was confirmed by using a 200-fold excess of unlabeled probe as a specific competitor. Protein–DNA complexes were separated by using a 6% non-denaturing acrylamide gel electrophoresis, and then transferred to positively charged nylon membranes and cross-linked in a cross-linker. Gel shifts were visualized with a streptavidin–horseradish peroxidase followed by the enhanced chemiluminescence detection system and Kodak X-OMAT Blue Autoradiography Film.

Protein content determination

The protein content was determined by the method of Bradford (31) with bovine serum albumin as a standard.

Statistical analysis

Data are indicated as mean ± SD of three different determinations. Differences between variants were analyzed by the Student's *t*-test for unpaired data. Values of $P < 0.05$ were regarded as statistically significant.

Results

LAB inhibits PMA-induced invasion of $HepG_2$ cells by suppressing the MMP-9 expression on a transcriptional level

In our previous study (29), we had demonstrated that no significant cytotoxicity was observed while treating $HepG_2$ cells with LAB at a concentration <100 μM for 24 h. The LAB could reduce PMA-induced MMP-9 activity of $HepG_2$ cells in a dose-dependent manner, but the MMP-2 activity was affected neither by PMA nor by LAB treatment. Here, quantitative analyses by a cell invasion assay with Matrigel-coated filter inserts showed that the invasion of $HepG_2$ cells was increased by ∼2.5-fold upon the PMA (200 nM) treatment

and the PMA-induced invasion was reduced by LAB treatment in a dose-dependent manner (Figure 1A). To further evaluate if the inhibitory effect of LAB on MMP-9 expression (Figure 1B) in $HepG_2$ cells is on the mRNA level, a semiquantitative RT–PCR analysis was performed. After treated with the LAB, mRNA level of MMP-9 was significantly reduced in a dose-dependent manner, whereas that of the internal control (glyceraldehyde-3-phosphate dehydrogenase) remained unchanged (Figure 1C). The results showed that the lucidenic acid might regulate the expression of MMP-9, at least partly, on the transcriptional level.

LAB inhibits PMA-induced MMP-9 activity of $HepG_2$ cells through suppressing phosphorylation of MAPK/ERK1/2

Since LAB was demonstrated to have inhibitory effects on the PMA-induced invasion as well as the transcriptional level of MMP-9 activity of $HepG_2$ cells, the effects of PMA and LAB on the expressions of MAPK and PI3K/Akt pathways were investigated by western blots to clarify the underlying mechanisms. The results of RT–PCR and western blots showed that PMA (200 nM) could induce the MMP-9 expression (Figure 1C) by the phosphorylation of ERK1/2 and Akt (Figure 2A and D), and LAB could inhibit the PMA-induced MMP-9 expression by suppressing the phosphorylation of ERK1/2 (Figure 2A) in $HepG_2$ cells. According to the densitometric analyses of blots versus the control, the PMA (200 nM) could induce 81.4, 82.4 and 29.4% of increases in ERK1, ERK2 and Akt phosphorylation, respectively. While compared with the blots of PMA treatment only, an additional treatment of LAB (50 μM) resulted in 49.8 and 46.2% decrease in the ERK1 and ERK2 phosphorylation, respectively. The PMA and the LAB both could not affect the SAPK/JNK (Figure 2B) and p38 MAPK (Figure 2C) significantly. To further confirm whether the PMA-induced and the LAB-inhibited MMP-9 expression were mainly through the ERK1/2-signaling pathways, $HepG_2$ cells were pretreated with MEK inhibitors (PD98059 or U0126; 0, 1, 2, 5, 10 or 20 μM) for 30 min and then stimulated with 200 nM PMA in the presence or absence of LAB (50 μM) for 24 h. The gelatin zymography analyses showed that the treatment of PD98059 (5 μM) or U0126 (1 μM) reduced the PMA-induced MMP-9 activities by 37.1 and 70.3%, respectively (Figure 3A). The combination treatment of PD98059 (5 μM) or U0126 (1 μM) with LAB (50 μM) could even reduce the PMA-induced MMP-9 activities by 53.0 and 80.6%, respectively (Figure 3A).

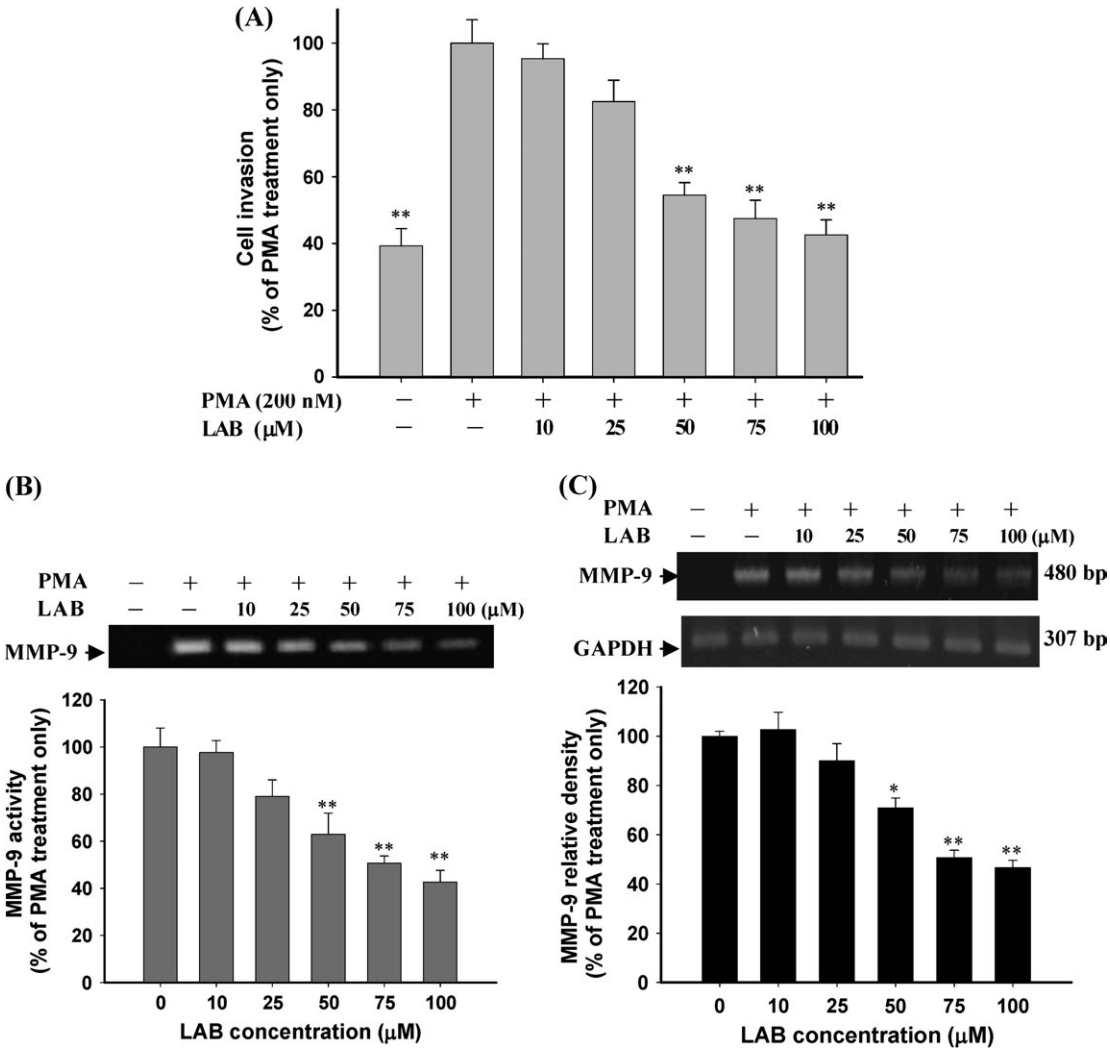

Fig. 1. Concentration-dependent inhibitory effects of LAB on PMA-induced invasion, MMP-9 activity and MMP-9 mRNA expression of $HepG_2$ cells. $HepG_2$ cells were treated with various concentrations of LAB (0, 10, 25, 50, 75 or 100 μM) for 24 h in the presence of 200 nM PMA. (**A**) The invasion ability of $HepG_2$ cells was determined by a Matrigel invasion assay. (**B**) MMP-9 activity of $HepG_2$ cells was determined by gelatine zymography. (**C**) The RNA extracted from $HepG_2$ cells was subjected to a semiquantitative RT–PCR. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. The final PCR products were quantified by densitometric analysis with that of PMA treated being 100%. Data are the mean ± SD of three independent experiments. Results were statistically analyzed with Student's *t*-test ($^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the PMA treatment only).

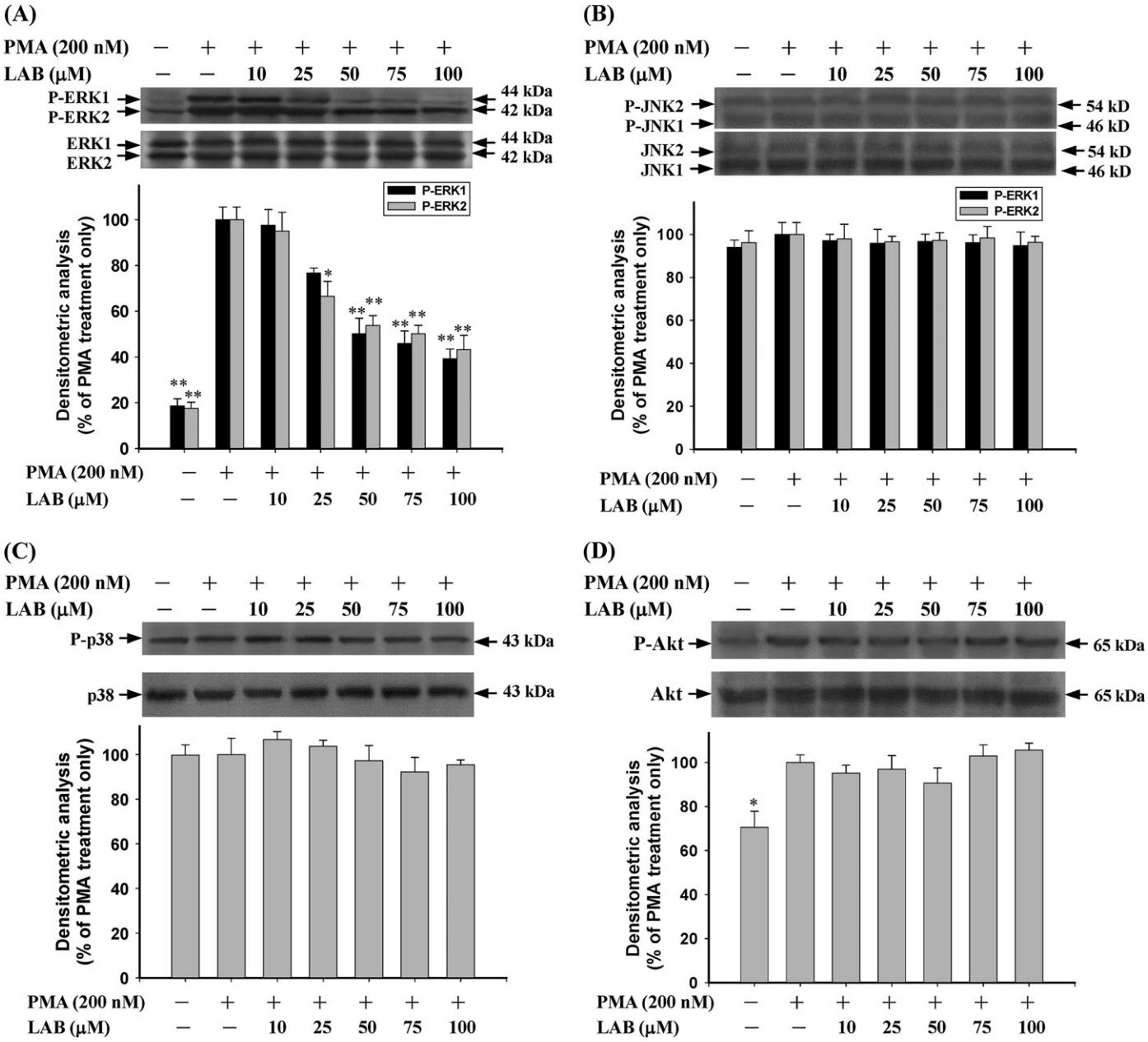

Fig. 2. PMA-induced and LAB-inhibited effects on the MAPK and PI3K/Akt signalings. $HepG_2$ cells were cultured in serum-free media containing 200 nM PMA and various concentrations of LAB (0, 10, 25, 50, 75 or 100 μM) for 24 h, and then the cell lysates were subjected to SDS–PAGE followed by western blots with anti-ERK1/2 (A), anti-JNK1/2 (B), anti-p38 (C) and anti-Akt (D) (total and phosphorylated) antibodies as described in Materials and Methods. Determined activities of these proteins were subsequently quantified by densitometric analyses with that of PMA treatment only being 100%. Data represent the mean ± SD of three independent experiments. Results were statistically analyzed with Student's *t*-test ($^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the PMA treatment only).

These results suggested that the induction of MMP-9 expression of $HepG_2$ cells by PMA could be partly through the activation of ERK1/2 and PI3K/Akt, and the inhibition of MMP-9 expression of $HepG_2$ cells by LAB could be partly through the inactivation of ERK1/2. Since ERK1/2 was involved in the MMP-9 expression of $HepG_2$ cells, PKC was put into investigation to determine whether PKC is involved in this signaling pathway. Again, $HepG_2$ cells were pretreated with PKC inhibitor (GF109203X; 0, 1, 2, 5, 10 or 20 μM) for 30 min and then stimulated with PMA (200 nM) in the presence or absence of LAB (50 μM) for 24 h. The result showed that the treatment of GF109203X at a concentration as low as 1 μM in $HepG_2$ cells was fully inhibited the PMA-induced MMP-9 activity (Figure 3B). The phenomenon suggested that PKC might be essential for the regulation of PMA-induced MMP-9 expression.

LAB inhibits the invasion of $HepG_2$ cells through inactivation of MAPK/ERK1/2-signaling pathway

We have found that the LAB could inhibit the PMA-induced invasion of $HepG_2$ cells by suppressing the MMP-9 expression (Figure 1) through regulating the phosphorylation of ERK1/2 (Figure 2). Here, we further examined whether LAB inhibited the PMA-induced invasion of $HepG_2$ cells mainly through inhibiting phosphorylation of ERK1/2. $HepG_2$ cells were pretreated with MEK inhibitors (PD98059, 5 μM, or U0126, 1 μM) for 30 min and then stimulated with 200 nM PMA in the presence or absence of LAB (50 μM) for 24 h. The pretreatment of $HepG_2$ cells with PD98059 or U0126 decreased the PMA-stimulated cell invasion by 34.2 and 54.3%, respectively, and the combination treatment of PD98059 or U0126 with LAB could reduce the

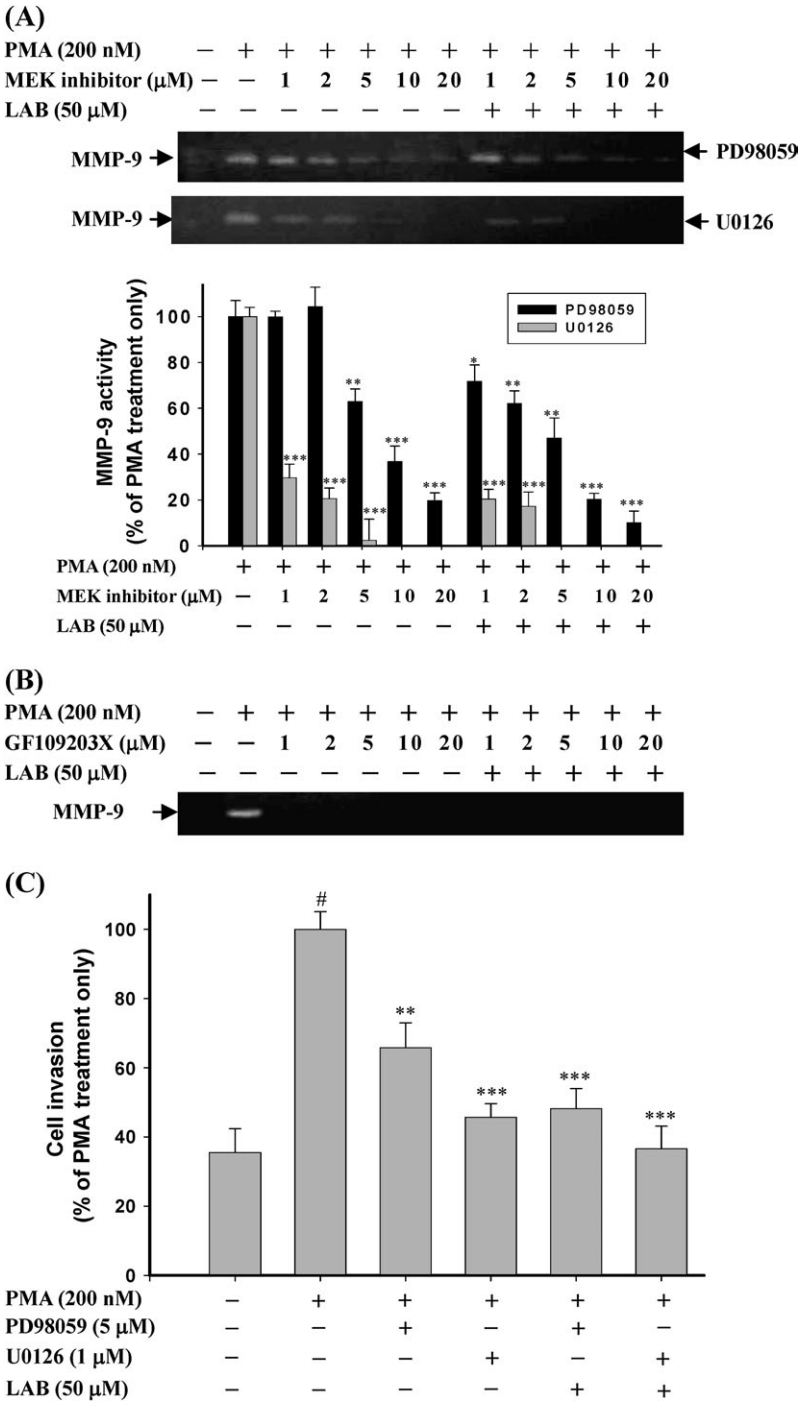

Fig. 3. Effects of MEK inhibitors (PD98059 and U0126), PKC inhibitor (GF109203X) and LAB on the MMP-9 activity and cell invasion of $HepG_2$ cells. $HepG_2$ cells were cultured in 24-well plates and pretreated with various concentrations (0, 1, 2, 5, 10 or 20 μM) of (**A**) PD98059 and U0126 and (**B**) GF109203X for 30 min. The cells were then stimulated with 200 nM PMA and incubated in the presence or absence of LAB (50 μM) for 24 h. The culture media were subjected to gelatin zymography to analyze the MMP-9 activity as described in Materials and Methods. Determined activities of these proteins were subsequently quantified by densitometric analyses with that of PMA treatment only being 100%. (**C**) $HepG_2$ cells were cultured in 24-well plates and pretreated with PD98059 (5 μM) or U0126 (1 μM) for 30 min. The cells were then stimulated with 200 nM PMA in the presence or absence of LAB (50 μM) for 24 h. The total number of cells that invaded the lower surface of the filter was counted. Data represent the mean ± SD of three independent experiments. Results were statistically analyzed with Student's *t*-test ($^{\#}P < 0.001$ compared with the control; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the PMA treatment only).

PMA-induced cell invasion by 51.8 and 63.4%, respectively, as compared with that of PMA treatment only (Figure 3C).

LAB inhibits transcriptional activity of MMP-9 gene through suppression of PMA-stimulated NF-κB and AP-1 activities

It was known that the expression of *MMP-9* gene is regulated through the transcriptional level interaction of AP-1 and NF-κB with their binding sequences in the *MMP-9* gene promoter. In the present study, we examined the effects of PMA and LAB on the transcriptional activities of NF-κB and AP-1 in $HepG_2$ cells. $HepG_2$ cells were treated with various concentrations of LAB (0, 10, 25, 50, 75 or 100 μM) in the presence of PMA (200 nM) for 24 h, and the nuclear extracts were analyzed by the EMSA for NF-κB and AP-1 DNA-binding activities. As shown in Figure 4, the NF-κB and AP-1 DNA-binding

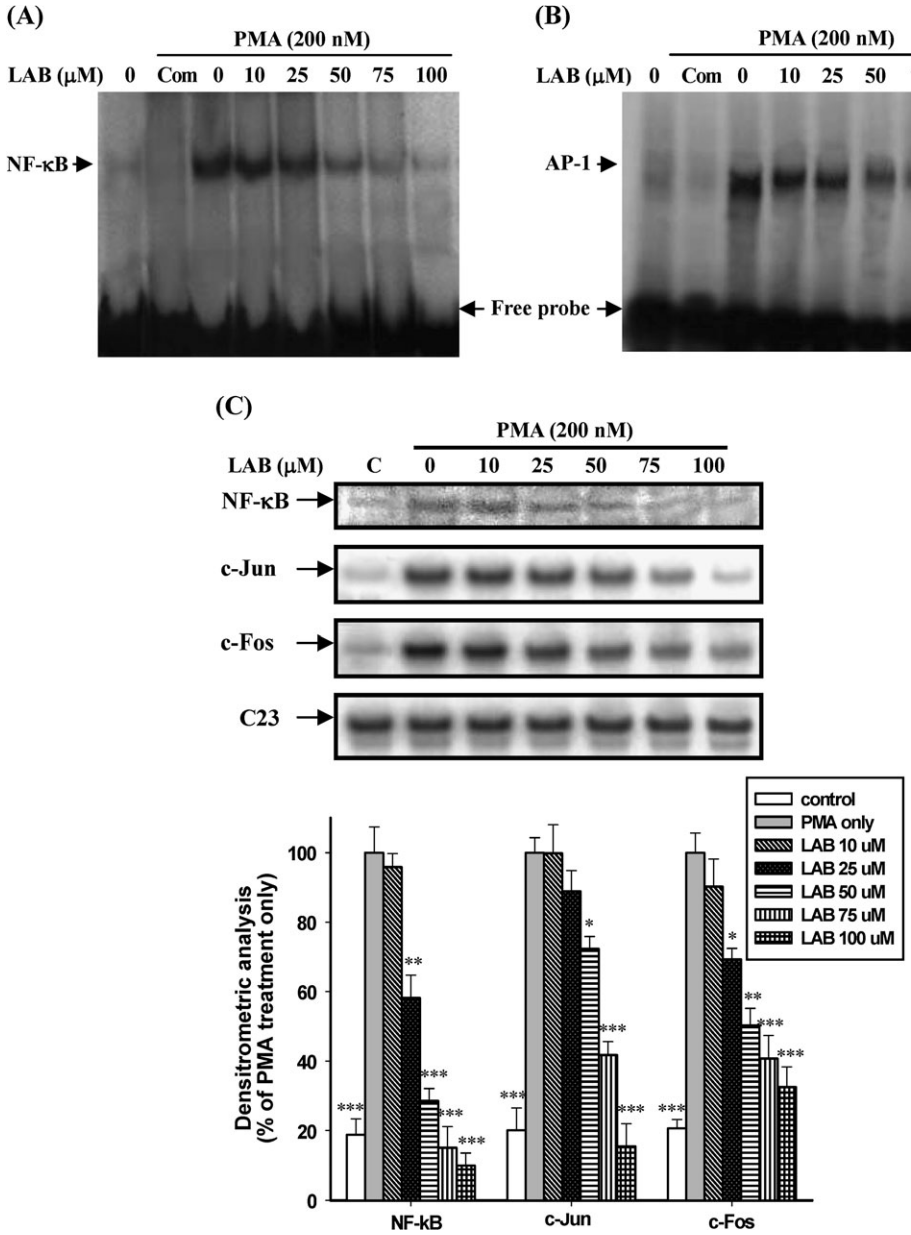

Fig. 4. Inhibitory effects of LAB on NF-κB and AP-1 DNA-binding activities and the expressions of NF-κB, c-Jun and c-Fos. Nuclear extracts were prepared from $HepG_2$ cells that treated with LAB at a concentration of 0, 10, 25, 50, 75 or 100 μM in the presence of PMA (200 nM) for 24 h, and then used to analyze (**A**) NF-κB and (**B**) AP-1 DNA-binding activity by EMSA, as described in Materials and Methods. Lane 1 represents nuclear extract from $HepG_2$ cells in the absence of PMA (negative control). Lane 2 (Com) represents nuclear extract from $HepG_2$ cells in the presence of PMA and incubated with unlabeled probe (competitor) to confirm the specificity of binding. (**C**) Nuclear extracts were subjected to SDS–PAGE followed by western blotting with anti-NF-κB, anti-c-Jun, anti-c-Fos and anti-C23 antibodies as described in Materials and Methods. Determined activities of these proteins were subsequently quantified by densitometric analyses with that of PMA treatment only being 100%. Data represent the mean ± SD of three independent experiments. Results were statistically analyzed with Student's *t*-test ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the PMA treatment only).

activities were dramatically increased by PMA (200 nM) treatment, and the PMA-stimulated NF-κB and AP-1 DNA-binding activities were strongly inhibited by LAB at the concentration >50 μM. Furthermore, the expressions of NF-κB, c-Jun and c-Fos in nuclear extracts were analyzed by western blotting. As a result, the protein levels of NF-κB, c-Jun and c-Fos in nuclear extracts were decreased by LAB treatment in dose-dependent manners.

It was known that the activation of NF-κB is through the phosphorylation of IκBα to release the NF-κB subunits (p65 and p50). The activated NF-κB is translocated from cytosol into the nucleus to regulate gene expression at a transcriptional level. As the phosphorylated IκBα level increased, in the meantime, the non-phosphorylated IκBα level decreased. The results reflected that the dimers of p65 and p50 subunits were released and the activated NF-κB level was increased. The IκB kinase (IκK) inhibitor, which blocks the phosphorylation of IκBα and non-phosphorylated specified IκBα antibody were used to examine the involvement of NF-κB on MMP-9 expression. $HepG_2$ cells were pretreated with various concentrations of Iκk inhibitor (0, 10, 25, 50 or 75 μM) for 1 h and then stimulated with 200 nM PMA in the presence or absence of LAB (50 μM) for 24 h. The culture media were subjected to gelatin zymography. The results showed in Figure 5A indicated that Iκk inhibitor dose dependently inhibited the PMA-induced MMP-9 expression and the combination treatment of Iκk inhibitor with LAB synergistically reduced PMA-induced MMP-9

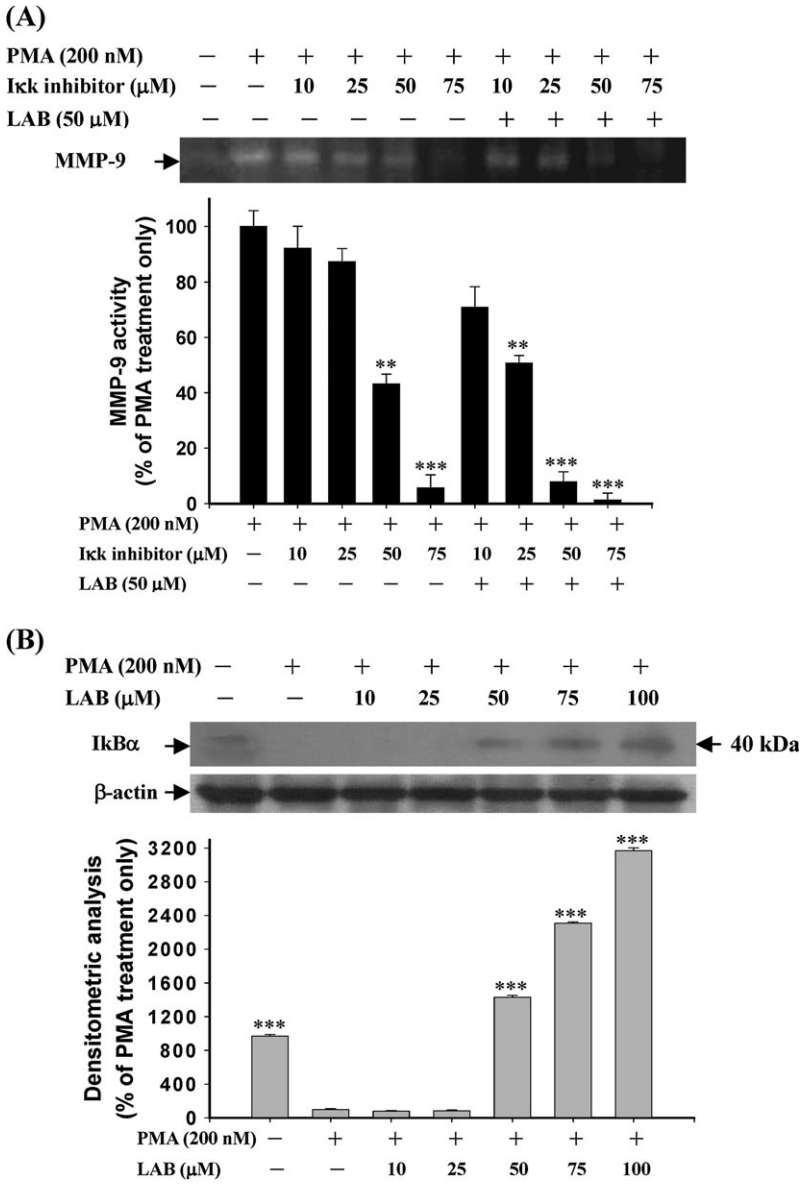

Fig. 5. Effects of IκB kinase (Ikk) inhibitor and LAB on the MMP-9 activity and IκBα protein expression of $HepG_2$ cells. (**A**) $HepG_2$ cells were cultured in 24-well plates and pretreated with various concentrations of Ikk inhibitor (0, 10, 25, 50 or 75 μM) for 1 h, and then stimulated with 200 nM PMA in the presence or absence of LAB (50 μM) for 24 h. The culture media were subjected to gelatin zymography to analyze the activity of MMP-9 as described in Materials and Methods. (**B**) $HepG_2$ cells were incubated with various concentrations of LAB (0, 10, 25, 50, 75 or 100 μM) in the presence or absence of 200 nM PMA for 24 h. The protein extracts were subjected to western blots as described in Materials and Methods. Determined activities and concentrations of these proteins were subsequently quantified by densitometric analyses with that of PMA treatment only being 100%. Data represent the mean ± SD of three independent experiments. Results were statistically analyzed with Student's *t*-test ($^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the PMA treatment only).

expression. The intensity of western blots reflected that the LAB at a concentration >50 μM could enhance IκBα protein expression (Figure 5B).

Discussion

Ganoderma lucidum has been used to reduce the invasion and metastasis at various types of cancers, including hepatoma, for many years. The bioactive ingredients and mechanisms underlying anti-invasion and antimetastasis of this mushroom are still under investigation by many researchers. We for the first time study the isolation of an effective MMP-9 inhibitory ingredient from *G.lucidum* and investigate the inhibitory molecular mechanisms on $HepG_2$ cells. The relationship between MMP expression and invasion in various cancers has been well documented. It was reported that PMA stimulated the migration and invasion of HCC 7721 cells (32) and that PMA induced MMP-9 expression in an HCC Malavu cell line (33). However, the mechanism of regulating MMP-9 expression in HCC cells is still poorly understood. In HCC SNU-387 and SNU-398 cell lines that do not secrete MMP-9 ordinarily, PMA treatment has been shown to stimulate the MMP-9 secretion in a dose-dependent manner without affecting the MMP-2 secretion (34). Our previous results also showed that PMA could induce MMP-9 activity but not MMP-2 activity of $HepG_2$ cells (29). The PMA-induced invasion and MMP-9 mRNA expression of $HepG_2$ cells could be reduced by the treatment with LAB (Figure 1). The findings from Chung *et al.* (35) also showed that the inhibition of PMA-induced MMP-9 transcriptional activity in $HepG_2$ cells treated by caffeic acid and caffeic acid phenyl ester. Such inhibition of the LAB on PMA-induced MMP-9 expression might provide a reasonable explanation for the inhibition of invasion on

$HepG_2$ cells. Clarifying the mechanism associated with the anti-PMA-induced invasion of LAB might help to prevent invasion and metastasis for a wide range of tumors.

It was known that activation of one or more MAPK pathways (e.g. ERK1/2, JNK and p38) is important for the MMP-9 induction by PMA in various cell types (21,36–38). In our present data, PMA induced MMP-9 expression of $HepG_2$ cells (Figure 1C) along with a strongly stimulated phosphorylation of ERK1/2 (Figure 2A) and a slightly stimulated phosphorylation of Akt (Figure 2D). While treated with specific inhibitors for ERK1/2 (PD98059 or U0126), PKC (GF109203X) (Figure 3), as well as PI3K (LY294002) (data not shown) on $HepG_2$ cells, the MMP-9 activities reduction were also observed (Figure 3). Walker *et al.* (39) has reported that PMA treatment is able to direct activation of PI3K and loss of PI3K protein expression or activity could prevent PMA-induced activation of cSrc and subsequent migration potential. PI3K might play an important role in facilitating co-localization between AFAP-110 and cSrc in response to PMA. Our results in Figure 2 also indicated that PI3K/Akt is involved in the PMA-induced MMP-9 activity of $HepG_2$ cells. However, it seems that treatments of PD98059, U0126 (Figure 3A) or LY294002 (data not shown) could not completely inhibit the PMA-induced MMP-9 activity and treatment of PKC inhibitor (GF109203X) completely abolished the PMA-induced MMP-9 activity (Figure 3B). Taken together, these phenomena indicated that the ERK1/2 and PI3K/Akt are required, but not absolutely, and the PKC is essential for the PMA-induced MMP-9 activity of $HepG_2$ cells. The PKC-dependent ERK activation is absolutely required for the PMA-induced MMP-9 secretion in HCC SNU-387 and SNU-398 (34), and PMA only activated Akt slightly in H4IIE hepatoma cells (40). In the present study, our findings suggested that the PMA induced MMP-9 activity of $HepG_2$ cells by strongly activating PKC and ERK1/2 as well as slightly activating Akt.

According to the findings of signaling pathways of PMA-induced MMP-9 activity, we presumed that the inhibitory effect of LAB on PMA-induced MMP-9 activity of $HepG_2$ cells might be through inactivating the signaling pathways of PMA induction. The treatment of LAB on PMA-induced $HepG_2$ cells could inhibit the MMP-9 expression (Figure 1C) and the ERK1/2 phosphorylation (Figure 2A) without affecting the Akt phosphorylation (Figure 2D). The involvement of ERK1/2 was further supported by the use of MEK inhibitors (PD98059 or U0126) (Figure 3A). However, LAB inhibited PKC or not is uncertained, owing to the fact that there were no observable bands except one (PMA treatment only) in Figure 3B. It was shown previously that the MMP-9 expression decreased (or increased) was along with the cell invasion decreased (or increased) (Figure 1) in $HepG_2$ cells. Hence, the involvement of MAPK/ERK1/2 pathway in LAB-inhibited invasion of $HepG_2$ cell was further supported by Matrigel invasion assay with MEK inhibitors. As shown in Figure 3C, the inhibitory effects of cell invasion with the combination treatment of PD98059 or U0126 and LAB (51.8 and 63.4%) were stronger than those with the treatment of PD98059 or U0126 (34.2 and 54.3%) alone. Taken together, the inhibitory effect of LAB on PMA-induced MMP-9 activity might be through inactivating the phosphorylation of

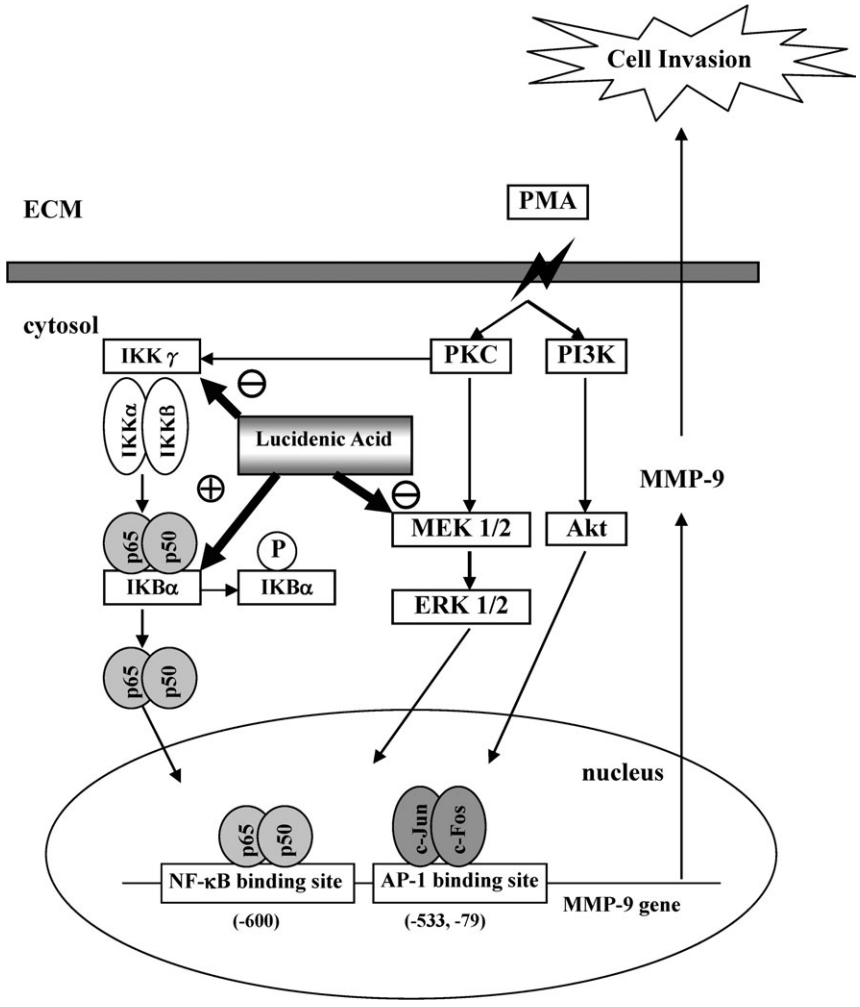

Fig. 6. Proposed signal transduction pathways by which PMA induced and LAB inhibited invasion of $HepG_2$ cells. 'O minus' means an inhibitory effect. 'O plus' means an enhanced effect.

ERK1/2 and contribute to the inhibition of cell invasion in $HepG_2$ cells.

The transcription of *MMP-9* gene is regulated by the upstream promoter sequence, including AP-1, NF-κB, stimulatory protein-1 and polyoma virus enhancer activator-binding sites (5,16). The AP-1 and NF-κB elements of MMP-9 promoter are centrally involved in the induction of the *MMP-9* gene associated with the invasion of tumor cells by PMA and cytokines. Thus, the regulation of AP-1 and NF-κB, the downstream of the MAPK and PI3K/Akt pathways, might be involved in PMA-induced and LAB-inhibited MMP-9 expression. We have demonstrated that the treatment of LAB on PMA-induced $HepG_2$ cells resulted in the inhibition of the AP-1 and NF-κB DNA-binding activities, and the nuclear translocation of c-Jun, c-Fos and NF-κB proteins were also reduced (Figure 4). AP-1 and NF-κB are involved in many pathological processes, such as angiogenesis, inflammation, metastasis and invasion (6,41,42). For instance, Cheng *et al.* (43) indicated that NF-κB modulates the radiation-enhanced MMP-9 activity in $HepG_2$ cells. Huang *et al.* (44) showed that the inhibitory effect of carnosol on melanoma cell migration and invasion by reducing MMP-9 expression is mediated through suppressing the ERK1/2, Akt, p38 and JNK pathways as well as inhibiting NF-κB and AP-1 DNA-binding activities. Chung *et al.* (35) also found caffeic acid and caffeic acid phenyl ester inhibit the transcriptional activity of MMP-9 in the PMA-induced $HepG_2$ cells by blocking NF-κB activation. Our findings indicated that the MMP-9 expression of $HepG_2$ cells by PMA and LAB treatment might also be regulated by the AP-1 and NF-κB DNA-binding activities. NF-κB is a heterodimeric protein consisting of p65 and p50 subunits, inactivated by non-phosphorylated IκBα binding and activated when IκBα is being phosphorylated. Futakuchi *et al.* (45) have shown that nuclear NF-κB activity of rat HCC is increased along with IκBα protein expression being suppressed by test compound treatment. By zymography and western blot analyses, the effects of Iκk inhibitor and LAB on the MMP-9 activity showed that the LAB inhibited MMP-9 expression through preventing IκBα being phosphorylated (Figure 5A) and enhancing IκBα protein expression (Figure 5B), both leading to inactivation of NF-κB DNA-binding activity.

In conclusion, we demonstrated that the LAB could effectively inhibit the PMA-induced invasion of $HepG_2$ cells; therefore, the LAB could be an anti-invasive bioactive ingredient in *G.lucidum*. Figure 6 shows the proposed mechanisms for the PMA-induced and LAB-inhibited invasion of $HepG_2$ cells. The anti-invasive effects of the LAB on the PMA-induced $HepG_2$ cells might be through inhibiting the phosphorylation of ERK1/2 and the activity of Iκk, as well as enhancing IκBα protein expression to reduce AP-1 and NF-κB DNA-binding activities, leading to the downregulation of MMP-9 expression. With the clarification of signal transduction mediators and transcriptional factors involved in the LAB anti-invasive process on human hepatoma cell line, it might be possible to develop specific mediators to inhibit undesired cell invasion. The lucidenic acids could be further tested by *in vivo* model to justify if effective for prevention of hepatoma invasion or metastasis.

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