Gallic acid suppresses the migration and invasion of PC-3 human prostate cancer cells via inhibition of matrix metalloproteinase-2 and -9 signaling pathways

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Abstract. Epidemiological studies have demonstrated that a natural diet or consumption of fruits or vegetables can decrease the risk of cancer development. Cancer cells can migrate to and invade other organs or tissues that cause more difficulty to treat them and this also results in the need for treatments targeting multiple cellular pathways. Gallic acid (GA) has been demonstrated to possess multiple biological activities including anticancer function. However, no report exist on GA inhibited invasion and migration of human prostate cancer cells. We investigated the effects of migration and invasion in GA-treated PC-3 human prostate cancer cells with a series of *in vitro* experiments. Boyden chamber transwell assay was used to examine the migration and invasion of PC-3 cells. Western blotting, real-time PCR and gelatin zymography were used for determining the protein levels, gene expression and enzyme activities of matrix metalloproteinase-2 (MMP-2) and -9 *in vitro*. Results indicated that GA inhibited the invasion and migration of PC-3 cells and these effects are dose-dependent. GA inhibited the protein levels of MMP-2 and -9, son of sevenless homolog 1 (SOS1), growth factor receptor-bound protein 2 (GRB2), protein kinase C (PKC) and nuclear factor-κ B (NF-κB) p65, c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase 1/2 (ERK1/2), p38, p-AKT (Thr308) and p-AKT (Ser473), but it promoted the levels of phosphatidylinositol 3-kinase (PI3K) and AKT in PC-3 cells. GA also reduced the enzyme activities of MMP-2 and -9 in the examined cells. Moreover, the down-regulation of focal adhesion kinase (FAK) and Ras homolog gene family, member A (Rho A) mRNA expression levels, and up-regulation of the tissue inhibitor of metalloproteinase-1 (TIMP1) gene levels occurred in GA-treated PC-3 cells after 24 h treatment. Based on these observations, we suggest that GA might modulate through blocking the p38, JNK, PKC and PI3K/AKT signaling pathways and reducing the NF-κB protein level, resulting in the inhibition of MMP-2 and -9 of PC-3 human prostate cancer cells.

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Introduction

Prostate cancer is the most common malignancy and the second leading cause of cancer related death among men in the USA (1). In Taiwan, prostate cancer also is one of the major causes of cancer-related deaths in men and about 8 persons per 100,000 die annually from prostate cancer based on the report from Department of Health, Taiwan in 2009. That age and family history of prostate cancer are the major risk factors for prostate cancer (2). Furthermore, men with an affected father or brother are approximately twice as likely to develop the disease themselves (3). Prostate cancer has been shown to produces metastasis to the lymph nodes and bones, leading to morbidity and mortality (4,5).

Treat of patients with prostate cancer includes surgery, radiation, chemotherapy, or a combination of radiotherapy and chemotherapy, but the results are still unsatisfactory. Metastasis of cancer cells makes treatment difficult as it involves invasion and migration of tumor cells. In tumor cells, the matrix metalloproteinases (MMPs) are overexpressed and the MMPs are involved in the invasion and metastasis of various tumor cells (6-8). Thus, inhibition of MMP expression

and/or inhibition of the activities of MMP enzyme can be used as early targets for preventing cancer metastasis (9-11). Natural products from plants can inhibit cancer development (12) and can act as a cancer therapy agent (13) such as paclitaxel, which have been used in the clinic (14).

Gallic acid (3,4,5-trihydroxybenzoic acid, GA), a naturally occurring plant phenol, is abundant in natural plants (15-19) and it has been reported to have anti-bacterial (20), anti-viral (21), anti-inflammatory (16), antioxidant (22) and antitumor functions in many human cancer cell lines (21-23). Although GA has been shown to inhibit the growth of prostate cancer cells, there is no available information addressing GA-inhibited migration and invasion of prostate cancer cells *in vitro*. Therefore, in the present study, we focused on the *in vitro* anti-migration and anti-invasion of GA on human prostate cancer PC-3 cells and investigated the possible signaling pathways *in vitro*.

Materials and methods

Materials and reagents. Gallic acid (GA), propidium iodide (PI) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). RPMI-1640 medium, L-glutamine, fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Primary antibodies MMP-2, MMP-9, SOS1, GRB2, PKC, NF-κB p65, JNK1/2, ERK1/2, p38, PI3K, AKT, p-AKT (Thr308), p-AKT (Ser473), β-actin and second antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and diluted in PBS Tween-20 before used for Western blot analysis.

Cell culture. The human prostate cancer PC-3 cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). PC-3 cells were immediately cultured onto 75 cm^2 tissue culture flasks with RPMI-1640 medium with 2 mM L-glutamine were adjusted to contain supplemented with 10% FBS, 100 Units/ml penicillin and 100 μg/ml streptomycin and grown at 37˚C under a humidified 5% CO_2 atmosphere.

Flow cytometric assay for cell viability. Approximately $2x10^5$ cells/well of PC-3 cells were cultured in 12-well plates for 24 h, and then various concentrations of GA were added to each well for final concentrations of GA at 25, 50, 100 and 150 μM, and 0.5% DMSO (as a control) for 24 and 48 h. Cells were harvested from each treatment were stained with PI (5 μg/ml), and then analyzed by flow cytometry as previously described (24). These cells were analyzed with a flow cytometer (BD FACSCalibur, San Jose, CA, USA) equipped with an argon ion laser at 488 nm wavelength (24,25).

In vitro wound closure. Approximately $2x10^5$ cells/well of PC-3 cells were cultured in a 12-well plate for 24 h wounding by scratching with a pipet tip, and cells in the each well were incubated with serum-free RPMI-1640 medium and treated with or without GA (0, 25 and 50 μM) for 24 h. Cells were photographed using a phase-contrast microscope (x200) as previously described (26,27).

In vitro invasion assay. The invasion assay of PC-3 cells was performed by using Matrigel (BD Biosciences, Bedford, MA, USA)-coated transwell cell culture chambers (8 μm pore size, EMD Millipore, Temecula, CA, USA) as described previously (31). PC-3 cells (10^4 cells/0.4 ml) were cultured for 24 h in serum-free RPMI-1640 medium and then placed in the upper chamber of the transwell insert ($5x10^4$ cells/well) and treated with 0.5% DMSO (as a control) or GA (25, 50 or 100 μM). However, the RPMI-1640 medium containing 10% FBS was placed in the lower chamber. All cells in each treatment were incubated for 24 or 48 h at 37˚C in a humidified atmosphere with 95% air and 5% CO_2. At the end of incubation, a cotton swab was used to remove the non-invasive cells maintained in the upper chamber and the invasive cells were fixed with 4% formaldehyde in PBS and stained with 2% crystal violet in 2% ethanol and cells were counted and photographed under a light microscope at x200 magnification (27,28).

In vitro migration assay. The migration assay of PC-3 cells was performed through the chemotactic directional migration by using a 24-well transwell insert. The 8 μm pore filters (EMD Millipore) were coated with 30 μg type I collagen (EMD Millipore) for 1 h and then the PC-3 cells (10^4 cells/0.4 ml RPMI-1640) were placed in the upper chamber and treated with 0.5% DMSO (as a control) or with or without GA (25, 50 and 100 μM) then to undergo migration for 24/48 h. At the end of incubation, a cotton swab was used to remove the non-migrated cells in the upper chamber then the filters were individually stained with 2% crystal violet and the migrated cells adherent to the underside of the filter were examined, counted and photographed under a light microscope at x200 magnification (26,28).

Cell-matrix adhesion assay. PC-3 cells ($2x10^5$ cells/well) were exposed to various concentrations of GA (25, 50 and 100 μM) for 24 and 48 h, and then seeded onto 24-well plates coated with 150 μl type I collagen (10 μg/ml) (EMD Millipore) overnight for 2 h. Afterward, non-adherent cells were removed and washed with PBS, and then adherent cells were fixed in 70% ethanol for 15 min. After being stained with 0.2% crystal violet for 10 min, fixed cells were washed and underwent lysed in 0.2% Triton X-100 for 30 min. The lysed solution (150 μl) was added to each well of the 96-well ELISA plates, and then the absorbance at 540 nm was measured as previously described (29,30).

Gelatin zymography. The activities of MMP-2 and -9 in PC-3 cells were measured after exposure to various concentrations of GA. PC-3 cells ($5x10^5$ cells/well) plated in 12-well tissue culture plates were incubated in serum-free RPMI-1640 medium with or without 25, 50, 100 and 150 μM GA for 24 and 48 h, and the conditioned medium was then collected. The collected individual medium was electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) containing 0.1% gelatin (Sigma-Aldrich Corp.). After electrophoresis, the gels were soaked in 2.5% Triton X-100 in dH_2O twice for a total of 60 min at 25˚C, then were incubated in substrate buffer (pH 7.6, 50 mM Tris, 10 mM $CaCl_2$, 50 mM and 0.05% Brij 35) at 37˚C for 18 h. Bands

corresponding to activity of MMP-2 and -9 were visualized by negative staining using 0.3% Coomassie Brilliant Blue in 50% methanol and 10% acetic acid as described elsewhere (27). Quantification of the data from the band density was performed by NIH ImageJ software.

Western blot analysis. To confirm whether or not GA-affected cell migration and invasion were through the inhibition of associated protein expression, PC-3 cells ($1x10^6$ cells/well) were placed in 6-well plates and incubated with or without GA (25 and 50 μM) for 24 and 48 h. Cells were harvested and resuspended in ice-cold 50 mM potassium phosphate buffer (pH 7.4) containing 2 mM EDTA and 0.1% Triton X-100 for sonication. The homogenate was centrifuged at 13000 g for 10 min at 4˚C to remove cell debris. The supernatant was collected and total protein was quantitated using a Bio-Rad protein assay kit (Hercules, CA, USA) with bovine serum albumin (BSA) as the standard. Protein abundance of MMP-2, MMP-9, SOS1, GRB2, PKC, NF-κB p65, JNK1/2, ERK1/2, p38, PI3K, AKT, p-AKT (Thr308) and p-AKT (Ser473) (Santa Cruz Biotechnology, Inc.) were determined by SDS-PAGE and Western blotting as previously described (24-26). NIH Image J software was used to determine the band intensity from Western blot analysis.

Real-time PCR of FAK, Rho A, ROCK1, TIMP1 and TIMP2. PC-3 cells ($1x10^6$ cells/well) were placed in 6-well plates and incubated without and with GA (25 or 50 μM) for 24 h. Cells were harvested and total RNA was extracted from each treatment by using the Qiagen RNeasy Mini Kit (Qiagen, Inc., Valencia, CA, USA) as described previously (24,25). RNA samples were reverse-transcribed at 42˚C with High Capacity cDNA Reverse Transcription Kit for 30 min according to the protocol of the supplier (Applied Biosystems, Carlsbad, CA, USA). The primer sets as FAK-F: TGAATGGAACCTCG CAGTCA; FAK-R: TCCGCATGCCTTGCTTTT; Rho A-F: TCAAGCCGGAGGTCAACAAC; Rho A-R: ACGAGC TGCCCATAGCAGAA; ROCK1-F: ATGAGTTTATTCCT ACACTCTACCACTTTC; ROCK1-R: TAACATGGCAT CTTCGACACTCTAG; TIMP1-F: TGTTTATCCATCCCC TGCAAA; TIMP1-R: CAAGGTGACGGGACTGGAA; GAPDH-F: ACACCCACTCCTCCACCTTT; TIMP2-F: GGGCCAAAGCGGTCAGT; TIMP1-R: TTGAACATCT TTATCTGCTTGATCTCA; GAPDH-F: ACACCCAC TCCTCCACCTTT; GAPDH-R: TAGCCAAATTCGTTG TCATACC. Quantitative PCR conditions were: 2 min at 50˚C, 10 min at 95˚C and 40 cycles of 15 sec at 95˚C; 1 min at 60˚C using 1 μl of the cDNA reverse-transcribed as described above, 2X SYBR Green PCR Master Mix (Applied Biosystems) and 200 nM of forward and reverse primers. Applied Biosystems 7300 Real-Time PCR system was used for each assay in triplicate and expression fold-changes were derived using the comparative CT method (25,27).

Statistical analysis. Statistical differences were performed by using the Student's t-test and considered significant at the $^*p<0.05$. All data are presented as the means ± SD of three experiments.

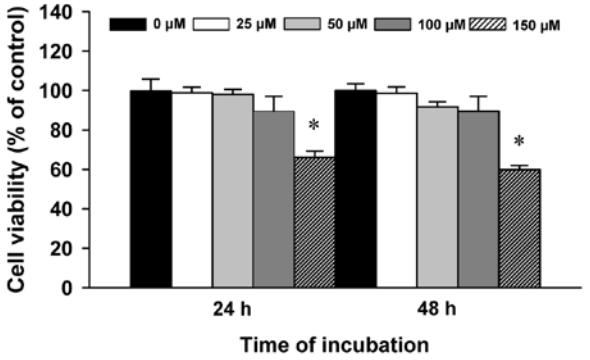

Figure 1. GA decreases the percentage of viable PC-3 cells *in vitro*. Cells were placed in 90% RPMI-1640 medium + 10% FBS with 0, 25, 50, 100 and 150 μM of GA for 24 and 48 h. The cells were collected and analyzed for viability by flow cytometry as described in Materials and methods. Each point is mean ± SD of three experiments. $^*p<0.05$, statistically significant when compared with the untreated control.

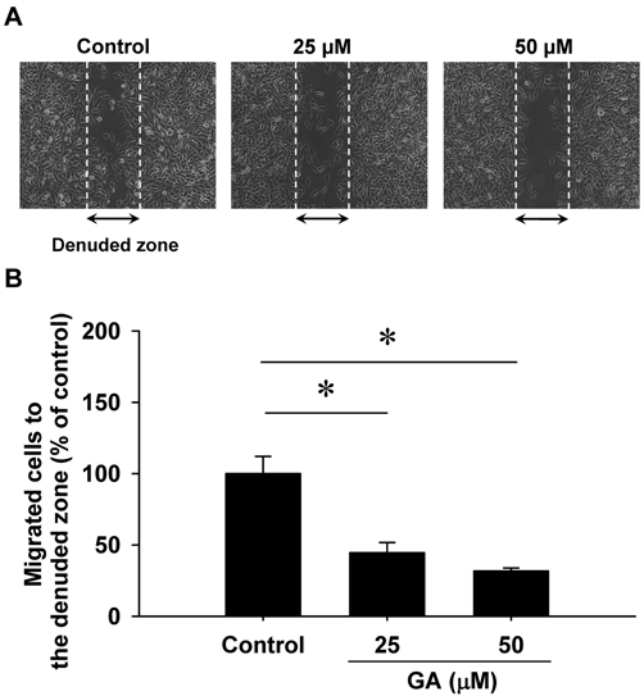

Figure 2. GA inhibits the *in vitro* wound closure of PC-3 cells. Confluent monolayer of PC-3 at a density of $2x10^5$ cells/well in 12-well plates was wounded with a scratch and rinsed by PBS to remove debris. Then, cells were incubated with or without GA (25 and 50 μM) for 24 h and 0.5% DMSO as control sample. The relative wound closures were monitored and photographed using a Nikon phase-contrast microscope (A). Fields shown are representative of the width of quadruplicate wounds and experiments were made in triplicate cultures. The percentage of inhibition was calculated (B). $^*p<0.05$, statistically significant when compared with the untreated control.

Results

GA decreases the percentage of viable PC-3 cells in vitro. The PC-3 cells were treated with various concentrations of GA for 24 and 48 h, and then cells from each treatment were collected for PI exclusion method for percentage of viability determination by flow cytometric assay. As shown in Fig. 1, there were

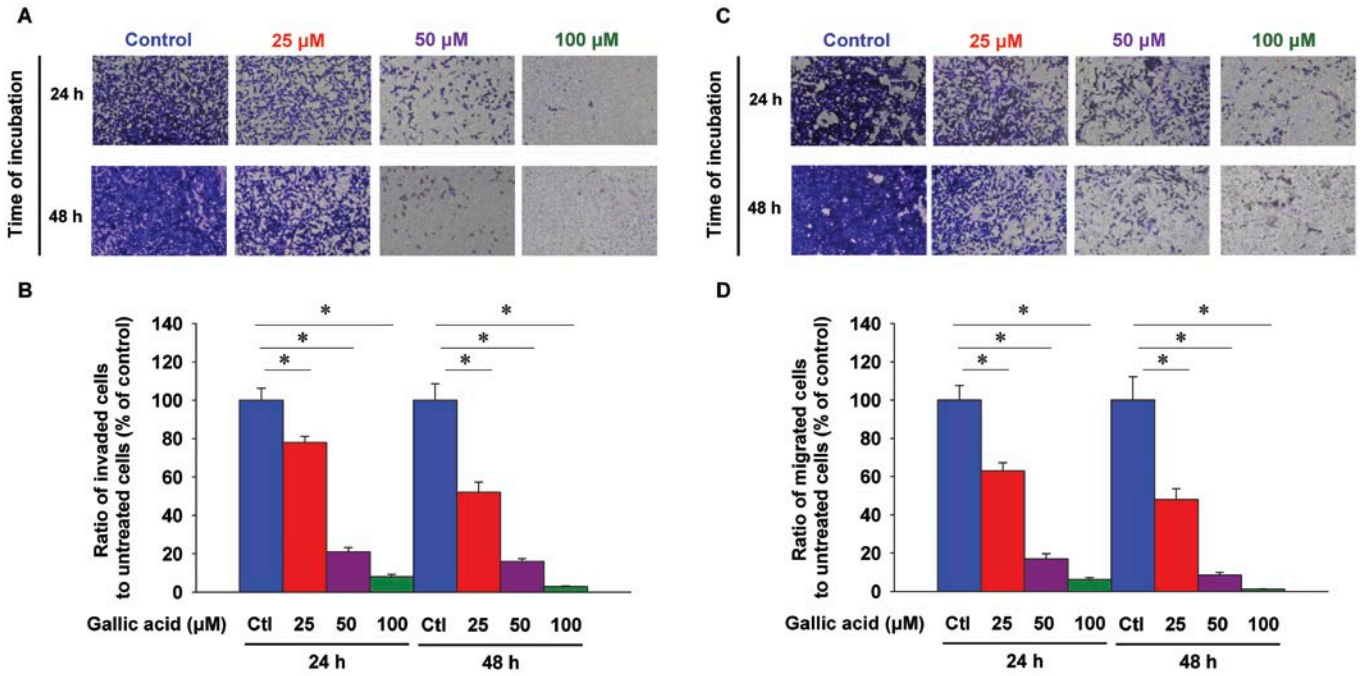

Figure 3. GA inhibits cell invasion and migration of PC-3 cells. Cells were treated with 25, 50 and 100 μM of GA or 0.5% DMSO as control sample (Ctl) for 24 and 48 h. (A) Cell invasion was examined in a Boyden chamber; polycarbonate filters (pore size, 8 μm) were precoated with Matrigel; (C) cell migration was examined in a Boyden chamber and Matrigel-coated transwell with type I collagen polycarbonate filters (pore size, 8 μm); invasion (B) and migration (D) ability of PC-3 cells were quantified by counting the number of cells that invaded the underside of the porous polycarbonate membrane under microscopy and represent the average of three experiments. *p<0.05, statistically significant difference between GA-treated groups and the control.

decreased the percentage of viable cells when compared to control groups (p<0.05) at 24- and 48-h treatment of 150 μM GA. Based on this result, there is no significant cytotoxic effect at <100 μM of GA in PC-3 cells.

GA inhibits the motility and in vitro wound closure of PC-3 cells. We investigated the effects of GA on migration of PC-3 cells by means of a wound closure assay. As shown in Fig. 2, relative wound closure decreased in control cells, but was higher than the GA treated cells. Fig. 2B indicates that the inhibition was at 44 and 74% when cells were incubated with GA at 25 and 50 μM for a 24-h treatment, respectively. These effects indicated that GA inhibited cell migration in a dose-dependent manner.

GA suppresses the invasion, migration and adhesion of PC-3 cells. To further confirm whether GA inhibited the migration of PC-3 cells, we investigated the effects of GA on invasion and migration of PC-3 cells *in vitro* and the results are shown in Fig. 3. The invasion assay indicates that PC-3 cells moved from the upper chamber to the lower chamber in the absence of GA (control group); however, the penetration of the Matrigel-coated filter by PC-3 cells was inhibited in the presence of GA (Fig. 3A). The percent inhibition at 25-100 μM was 22-90% and 53-96% (Fig. 3B) when PC-3 cells were incubated with GA for 24-h and 48-h treatment, respectively. The migration assay showed that GA had a significant inhibitory effect on cell migration at concentrations between 25-100 μM (Fig. 3C). Data in Fig. 3D indicate that the inhibition was 36-96% and 54-98% when cells were exposed to GA

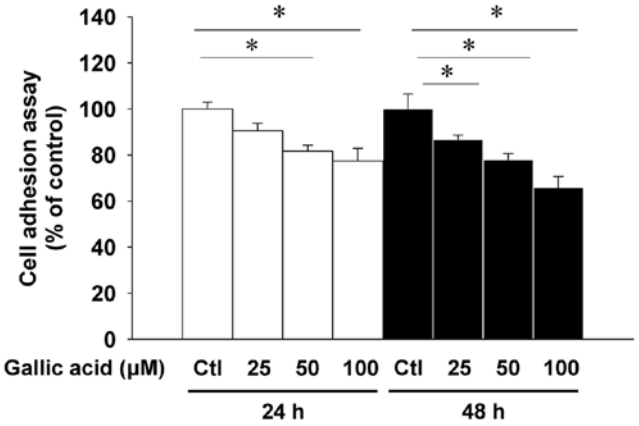

Figure 4. GA inhibited the adhesion of PC-3 cells. Cells were exposed to 25, 50 and 100 μM of GA for 24 and 48 h, and 0.5% DMSO as control sample (Ctl). The adhesion assay was performed on 24-well plates coated with collagen (EMD Millipore) using 2 h incubations as described in Materials and methods. The results are presented as the percentage of vehicle-treated control (Ctl). Each bar represents the mean ± SD of 3 independent experiments. *p<0.05, statistically significant when compared with the untreated control.

for 24 h and 48 h, respectively. As seen in Fig. 4, a significant inhibitory effect of cell adhesion was observed in GA-treated PC-3 cells when compared to the control cells. Furthermore, these inhibitory effects of GA on PC-3 cells are not due to the cytotoxic effect of GA because the viability of these cancer cells was barely affected by GA in 150 μM in the concentration range tested.

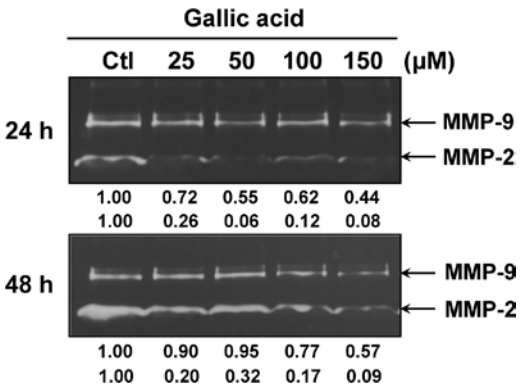

Figure 5. GA inhibits MMP-2 and MMP-9 activities in PC-3 cells. Cells ($5x10^5$ cells/well) were incubated with 25, 50, 100 and 150 μM of GA for 24 and 48 h, and 0.5% DMSO as control sample (Ctl). Cells were harvested and separated by gelatin zymography as described in Materials and methods. The ratios of MMP-2 and MMP-9 activities were quantitated.

GA attenuates the enzyme activities of MMP-2 and MMP-9 in PC-3 cells. We investigated whether GA could affect the secretion of MMP-2 and -9 in PC-3 cells. Gelatin zymography was used to analyze GA-affected MMP-2 and MMP-9 activities for 24 and 48 h treatment of GA (0, 25, 50, 100 and 150 μM). The results shown in Fig. 5 indicate that GA reduced MMP-2 and MMP-9 activities in a dose-dependent manner.

GA alters the levels of proteins and gene expression associated with migration and invasion in PC-3 cells. To investigate the effects of GA on the migration and invasion associated with the inhibition of associated proteins, we determined the effects of GA on the levels of proteins associated with migration and invasion in PC-3 cells and the results are presented in Fig. 6A-D, indicating that GA reduced protein levels of MMP-2 and -9 (Fig. 6A), SOS1, GRB2, PKC and NF-κB p65 (Fig. 6B), JNK1/2, ERK1/2 and p38 (Fig. 6C) and p-AKT (Thr308) and p-AKT (Ser473) (Fig. 6D) in PC-3 cells. However, GA

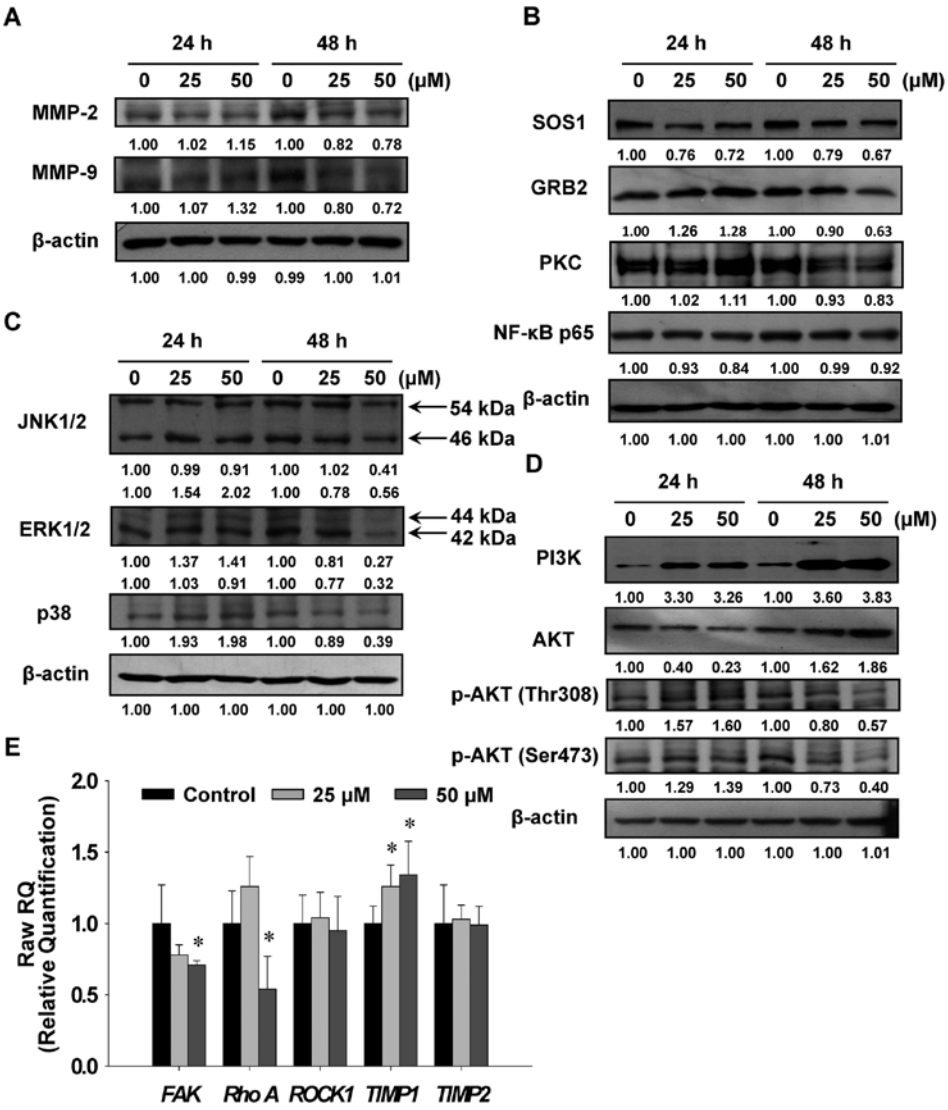

Figure 6. GA affects the migration and invasion-associated proteins and gene expression levels in PC-3 cells. Cells were treated with 0, 25 and 50 μM of GA for 24 and 48 h, and 0.5% DMSO as control sample (Ctl). The total proteins were collected and the proteins levels [(A) MMP-2, MMP-9; (B) SOS1, GRB2, PKC, NF-κB p65; (C) JNK1/2, ERK1/2, p38; (D) PI3K, AKT, p-AKT (Thr308) and p-AKT (Ser473)] were examined by SDS-PAGE and Western blotting as described in Materials and methods. Cells from each treatment were harvested and the total RNA was extracted and RNA samples were reverse-transcribed for real-time PCR as described in Materials and methods. The ratios of FAK, Rho A, ROCK1, TIMP1 and TIMP2 mRNA/GAPDH (E) are presented. Data represents mean ± SD of three experiments. $^*p<0.05$, statistically significantly when compared with the untreated control.

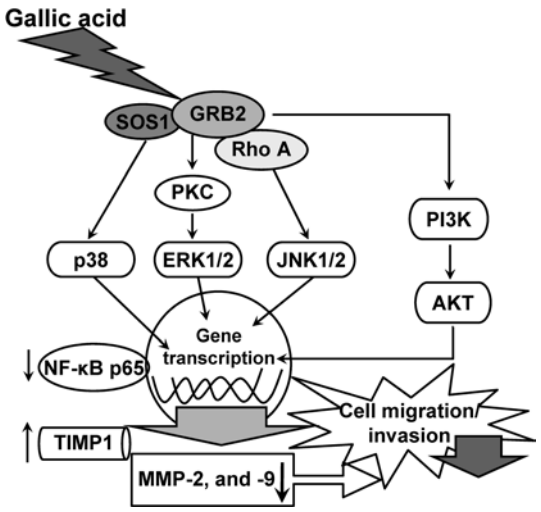

Figure 7. The proposed signaling pathways for GA-inhibited migration and invasion of PC-3 human prostate cancer cells *in vitro*.

promoted the levels of PI3K and AKT (Fig. 6D) in examined cells. In order to investigate whether or not GA affected migration- and invasion-associated gene expression in PC-3 cells, cells were treated with GA (25 and 50 μM) for 24 h and then FAK, Rho A, ROCK1, TIMP1 and TIMP2 mRNA expression were detected by real-time PCR. Results shown in Fig. 6E indicate that the mRNA expression levels of FAK and Rho A were down-regulated, and TIMP1 was up-regulated, but the levels of ROCK1 and TIMP2 were not significantly affects GA-treated PC-3 cells after 24 h exposure.

Discussion

It is well known that tumor metastasis occurs in many steps including vessel formation, cell attachment and adhesion, invasion, migration and cell proliferation, and these events are regulated by an extremely complex mechanism (31). Therefore, great attention is focused on developing agents or drugs that can inhibit metastasis; however, the anti-metastatic agents are still lacking (32). In the present study, we investigated the GA affects on invasion/migration of human prostate cancer PC-3 cells *in vitro* by using a Boyden chamber assay to quantify the migratory potential of PC-3 cells. The results indicated that GA is able to inhibit *in vitro* invasive and migration abilities (Fig. 3A and B) as well as decrease the adhesive capabilities (Fig. 4) in PC-3 cancer cells. Overall, our results indicate that GA decreased the metastasis of PC-3 cells *in vitro,* which was determined by adhesion, invasion and migration assays.

Numerous reports have been shown that the inhibition of MMP expression and/or inhibition of the activities of MMP enzymes can be used as early targets for preventing cancer metastasis (9,32-33). MMP-2 (72-kDa gelatinase A) and MMP-9 (92-kDa gelatinase B) are involved in the invasive metastatic potential of tumor cells (34). Our results from wound healing and invasion assays also showed that GA inhibited the invasion and migration (Figs. 2 and 3) and it also reduced cell adhesion (Fig. 4) in PC-3 cells *in vitro*. GA inhibited the activities of MMP-2 and -9 which are involved in degradation of extracellular matrix and play important roles in cancer cell migration and invasion (34-37). Our results demonstrated that anti-metastastic effects of GA were associated with the inhibition of enzymatically degradative processes of metastasis in PC-3 cells (Fig. 5). The present results suggest that GA may have efficacy, if delivered to the bone site at concentrations between 20-40 μM, thus preventing metastasis of bone cancer cells.

GA also inhibited associated protein levels such as MMP-2 and -9 (Fig. 6A), SOS1, GRB2, PKC and NF-κB p65 (Fig. 6B), JNK, ERK1/2 and p38 (Fig. 6C) and p-AKT (Thr 308) and p-AKT (Ser-473) (Fig. 6D). GA inhibited the levels of ERK1/2 which is the key molecules of the extracellular signal-regulated kinase (ERK) signaling pathway that has been shown to promote tumor invasion and metastasis (Fig. 6C). The ERK signaling pathway up-regulated the expression of MMPs (30). We thus tested the effect of GA on the ERK signaling pathway. We found that GA inhibited the phosphorylation of AKT (Fig. 6D) in PC-3 cells, indicating that GA can inhibit the AKT signaling pathway.

Furthermore, the results indicated that GA also decreased the levels of AKT, p-AKT and JNK1/2 (Fig. 6C and D) in PC-3 cells. This is in agreement with other report that integrins activate MAPK and PI3K, leading to activation of AKT (37). Our results also showed that GA inhibited the levels of AKT, FAK, ROCK 1 and Rho A that may lead to inhibit the levels of MMP-2 and -9 in PC-3 cells, contributing to the decrease in migration of GA-treated PC-3 cells. It was reported that PI3K activation stimulated the downstream target AKT which associated with cell invasion (38,39). Other investigators have pointed out that PI3K activation stimulated the downstream target AKT, and AKT plays various and important roles in cell invasion (38-40) and the activation of ERK and AKT are involved in the development of endometrial cancer (41,42). Herein, our findings showed that GA inhibited the protein levels of PI3K, AKT, PKC, NF-κB, MMP-2 and MMP-9 in PC-3 cells *in vitro*. Furthermore, the activation of PKC could lead to the translocation of the protein to membranes for affecting the expression of MMP-9 through NF-κB or Sp-1 via mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling pathways (32,43-46). Based on results from real-time PCR GA inhibited the gene expression of FAK and Rho A, but promoted TIMP1 mRNA level of PC-3 cells (Fig. 6E). TIMP1 has been shown to regulate MMP2 activity and numerous pieces of evidence have indicated a correlation between elevated TIMP1 levels and diminished MMP-2 activity and invasiveness (46-48).

Collectively, GA has multiple anti-metastatic activities and has the potential to be developed into an anti-metastatic agent for prostate cancer. The possible signal pathways for GA to inhibit migration and invasion in PC-3 cells may be through down-regulation of PKC, p38, ERK, JNK and PI3K/AKT signaling pathways and NF-κB resulting in inhibition of MMP-2 and MMP-9 (Fig. 7). The *in vivo* efficacy studies with GA should be done in animal models in future investigations.

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