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Gallic acid inhibits gastric cancer cells metastasis and invasive growth via increased expression of RhoB, downregulation of AKT/small GTPase signals and inhibition of NF-κB activity

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ABSTRACT

Our previous study demonstrated the therapeutic potential of gallic acid (GA) for controlling tumor metastasis through its inhibitory effect on the motility of AGS cells. A noteworthy finding in our previous experiment was increased RhoB expression in GA-treated cells. The aim of this study was to evaluate the role of RhoB expression on the inhibitory effects of GA on AGS cells. By applying the transfection of RhoB siRNA into AGS cells and an animal model, we tested the effect of GA on inhibition of tumor growth and RhoB expression. The results confirmed that RhoB-siRNA transfection induced GA to inhibit AGS cells' invasive growth involving blocking the AKT/small GTPase signals pathway and inhibition of NF-κB activity. Finally, we evaluated the effect of GA on AGS cell metastasis by colonization of tumor cells in nude mice. It showed GA inhibited tumor cells growth via the expression of RhoB. These data support the inhibitory effect of GA which was shown to inhibit gastric cancer cell metastasis and invasive growth via increased expression of RhoB, downregulation of AKT/small GTPase signals and inhibition of NF-κB activity. Thus, GA might be a potential agent in treating gastric cancer.

Introduction

Gastric carcinoma (GC), which is the second most common cause of cancer-related death in the world, is responsible for more than 700,000 deaths per year (Parkin et al., 2005). In Asian countries, such as Korea and China, GC is the leading cause of cancer death. Conventional therapies for advanced-stage GC include surgery, chemotherapy, and radiotherapy, but the prognosis for advanced-stage disease remains poor. More than 80% of patients with advanced GC have lymph node metastasis, and the remote lymph nodes, such as the para-aortic nodes, are involved in 20% of GC. Therefore, control of lymph node metastasis is the most important prognostic factor for the treatment of GC (Dent et al., 1988; Maruyama et al., 1989; Yonemura et al., 1991). Radiation therapy or chemotherapy does not significantly affect the length or quality of life of patients with advanced GC (Kelsen, 1994). Thus, novel therapies are needed to target the molecular alterations that lead to GC development as well as progression.

Flavonoids are common constituents of the human diet, present in most fruit and vegetables, and comprise several classes, including flavanones, flavonols, flavones and isoflavone. In several cases, flavonoids have been reported to possess anticancer potential (Lee et al., 2006). Gallic acid (GA), widely distributed in plants and foods, has various biological effects. The anticancer effects of GA on Calu-6 and A549 lung cancer cells in relation to reactive oxygen species and glutathione was reported recently (You and Park, 2010). In addition, GA, the major anticancer compound in *Toona sinensis* leaf extracts, was shown to be cytotoxic to DU145 prostate cancer cells, through generation of reactive oxygen species and mitochondria-mediated apoptosis (Chen et al., 2009).

Our recent study showed that the inhibitory effects of GA on AGS cells may be partly exerted through the Ras/PI3K/AKT signaling

Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; GA, gallic acid; MMP, matrix metalloproteinases; TRITC, Tetramethylrhodamine B isothiocyanate; NF-κB, Nuclear factor-kappa B; I-κB, inhibitor protein-kappa B; DAPI, 4′-6-Diamidino-2-phenylindole.

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pathway (Ho et al., 2010). In addition, the increased protein levels of cytoplasmic IκB, which exert inhibitory effects on the transcriptional factor NF-κB, subsequently decrease MMP-2 and MMP-9 activities, resulting in antimetastatic effects. A noteworthy finding in our previous experiment was the increased RhoB expression in GA-treated cells. Members of the Rho family of small GTPases are key regulators of actin reorganization, cell motility, cell–cell and ECM adhesion as well as of cell cycle progression, gene expression, and apoptosis (Fritz and Kaina, 2006). RhoA, like other GTPase family members, such as Ras, Rac1, and cdc42, promotes oncogenesis, invasion, and metastasis (Khosravi-Far et al., 1995), however, emerging evidence points to a tumor-suppressive role for RhoB (Chen et al., 2000). Although Rho family GTPases RhoA, RhoB and RhoC share more than 85% amino acid sequence identity, they may play distinct roles in tumor progression. RhoA and RhoC have been suggested to have positive effects on tumor progression, but the role of RhoB in cancer, particularly in gastric cancer, remains unclear (Zhou et al., 2011).The aim of this study was to evaluate the role of RhoB expression on the inhibitory effects of GA on AGS cells by applying the transfection of RhoB siRNA into AGS cells. In addition, using an animal model, we tested the effect of GA on inhibition of tumor growth and RhoB expression.

Materials and methods

Materials. Gallic acid, DMSO, SDS, phenylmethylsulfonyl fluoride, bovine serum albumin (BSA), leupeptin, deoxycholic acid, and sodium orthovanadate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate buffer solution (PBS), trypsin-EDTA, and powdered Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco/BRL (Gaithersburg, MD, USA). Antibodies against AKT, PI3K, NF-κB, IκB, JNK/phospho-JNK, Jun/phosphor-Jun, Fos, Ras, Rac1, cdc42, fas, RhoA, and RhoB were obtained from BD Transduction Laboratories (San Diego, CA) and Santa Cruz Biotech (Santa Cruz, CA).

Cell culture. A human gastric carcinoma AGS cell line was obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan, ROC). AGS cells were maintained in F-12 nutrient mixture medium (Gibco/BRL, Gaithersburg, MD). The cells were cultured at 37 °C in 5% CO_2 in medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL of penicillin and 100 mg/mL of streptomycin).

Transient transfection of RhoB siRNA into AGS cells. For transient transfection of siRNA, we used a StealthTM Select RNAi Set (Cat No. 1299003) purchased from Invitrogen Corporation (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. One day prior to transfection, AGS cells were seeded in six-well dishes (2×10^5 cells/well) in DMEM without antibiotics, and the cells were expected to be 60% confluent the next day. On the day of transfection, 150 pmol of Stealth RNAi was diluted into 50 μL reduced serum medium and mixed gently. Meanwhile, 1 μL of Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) was incubated with 50 μL of reduced serum medium at room temperature. Afterwards, diluted StealthTM RNAi and Lipofectamine™ 2000 were combined by mixing gently and incubated for 15 min at room temperature. This mixture was then added to cells and incubated at 37 °C in a humidified CO_2 incubator. After 72 h, transfected cells were counted and subjected to cell migration assay and Western blot. Control cells were exposed to the transfectant with negative control siRNA (Thermo Scientific, Dharmacon Product, Lafayette, CO; category No: D-001810-10-05) instead of RhoB siRNA.

Immunoblotting. AGS cells were transfected with negative control siRNA or RhoB siRNA then treated with or without 3.5 μM GA for 24 h. The protein levels of RhoB were analyzed by Western blotting. β-actin was used for equal loading. Western blotting was performed according to a previously described method (Shiah et al., 1999). In short, the cell lysates were denatured in a sample buffer containing SDS, and equal amounts of total protein were separated on 8–15% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with 5% nonfat dry milk, the membranes were incubated overnight at 4 °C with the primary antibodies as indicated. The following antibodies were used: antibodies against NF-κB, IκB, PI3K, AKT, Ras, cdc42, Rac1, RhoA, and RhoB (Santa Cruz Biotech, Santa Cruz, CA). The following day, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies, and detection was performed using an enhanced chemiluminescence (ECL) reagent (Amersham Life Science, Amersham, UK).

Reverse transcription-polymerase chain reaction for RhoB mRNA. AGS cells were seeded in 6-well culture plates (2×10^5 cells/well) and grown to 80% confluence. Cells were transfected with negative control siRNA or RhoB siRNA then treated with or without 3.5 μM GA for 24 h. Total RNA was isolated, and the mRNA expression was analyzed by RT-PCR. The cells were then lysed in 1 mL TRIzol reagent (Invitrogen, Carlsbad, USA) and RNA extracted following the manufacturer's recommendations. Total cellular RNA was used for reverse transcription of cDNA by a standardized technique (MBI Fermentas, Hanover, USA). Obtain cDNA was amplified using specific primers. RhoB (GenBank accession no. NM_004040.2; 591 bp) forward, 5′-ATGGCGGCCATCCGCAAG AAGC-3′, reverse, 5′-TCATAGCACCTTGCAGCAGTTG-3′; glyceraldehyde-3-phosphate dehydrogenase (GenBank accession no. NM_002046.3; 270 bp), forward, 5′-TTGGTATCGTGGAGGGACTCA-3′, reverse, 5′-TGTC ATCATATTTGGCAGGTT-3′). After pre-denaturation at 95 °C for 15 s, polymerase chain reaction (PCR) was carried out as the following program: Rho B: 94 °C for 15 s, 57 °C for 20 s, then 36 cycles of 72 °C for 15 s and 72 °C for 10 min; GAPDH: 30 cycle of 94 °C for 30 s,

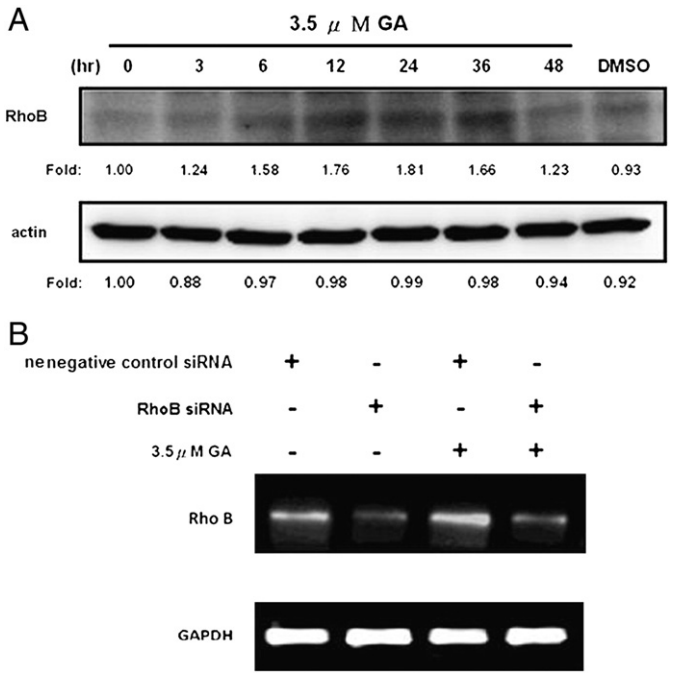

Fig. 1. Immunoblot analysis of the expression of RhoB in AGS cells treated with gallic acid (GA). (A) AGS cells were treated with or without 3.5 μM GA then harvested at different time (0, 3, 6, 12, 24, 36, 48 h). The protein levels of RhoB were analyzed by Western blotting. β-actin was used for equal loading. (B) Effects of gallic acid on RhoB mRNA level in AGS cell. AGS cells were transfected with control siRNA or RhoB siRNA then treated with or without 3.5 μM GA for 24 h. Total RNA was isolated, and the mRNA expression was analyzed by RT-PCR and the GAPDH as the loading control. Values were the average of triplicate experiments.

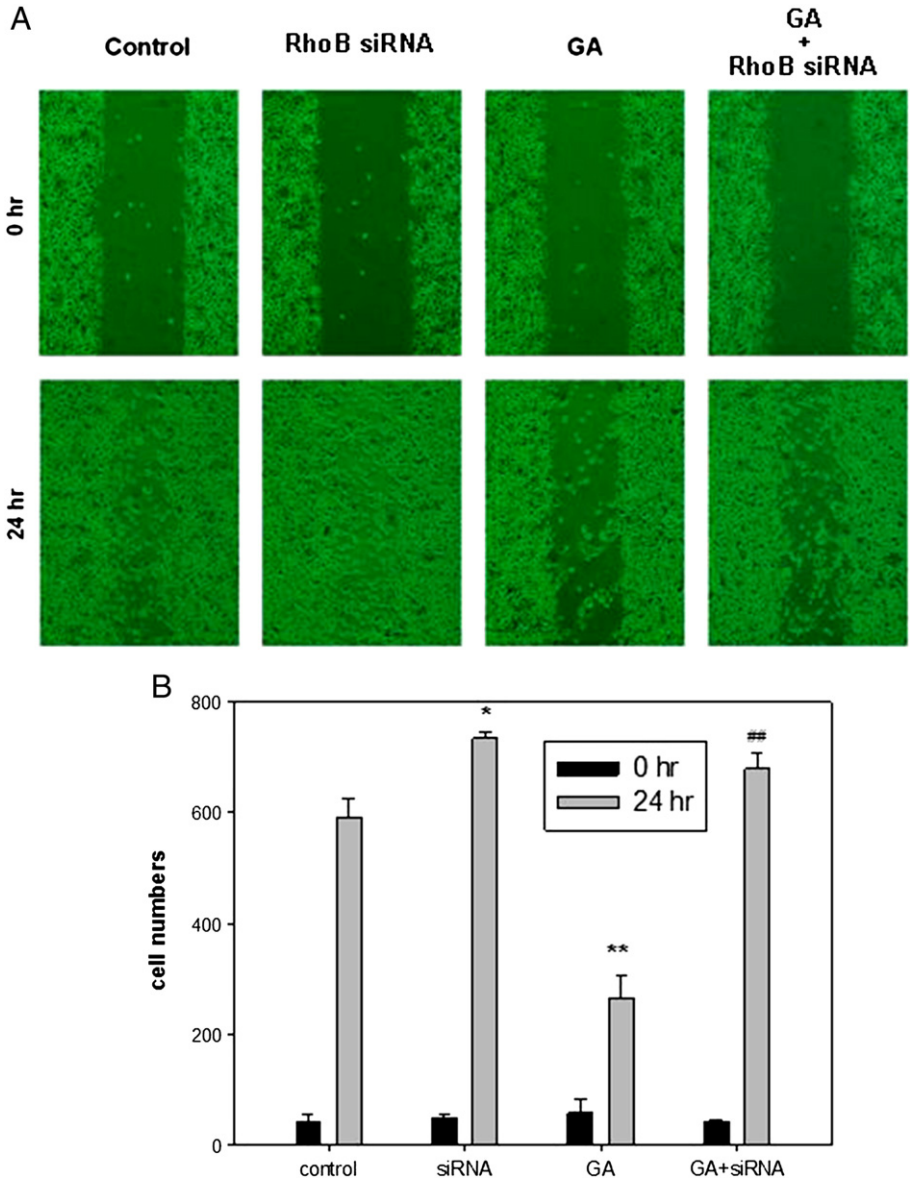

Fig. 2. Cell migration of AGS cells was inhibited by gallic acid (GA). (A) Monolayers of growth-arrested AGS cells were transfected with control siRNA or RhoB siRNA then treated with or without 3.5 μM GA .Cells were scraped and the number of cells in the denuded zone (i.e., wound) was assessed at 0 and 24 h under light microscopy. (B) Mean number of cells at I 0 and 24 h in the denuded zone and represents the average of three independent experiments as mean ± SD. (*$p<0.005$, **$p<0.0005$ compared with control, ##$p<0.0005$ compared with GA).

followed by annealing for 30 s at 55 °C, finally extended for 1 min at 72 °C. The PCR products were visualized on 2% agarose gel and captured by a digital camera.

Wound healing assay. To determine the cell motility, AGS cells were seeded in 6-well culture plates (1×10^6 cells/well) and grown to 80–90% confluence. Monolayers of growth-arrested AGS cells were transfected with negative control siRNA or RhoB siRNA then treated with or without 3.5 μM GA. The medium was aspirated, and a monolayer in the center of the cell was scraped with a sterile micropipette tip to create a denuded zone (gap) of constant width. Wound closure was monitored and photographed at 0 and 24 h by an Olympus CK-2 inverted microscope and an Olympus OM-1 camera. The cells that migrated across the white lines were counted in five random fields from each triplicate treatment, and data are presented as the mean ± SD.

Boyden chamber assay. The migration abilities of the AGS cells transfected with negative control siRNA or RhoB siRNA and then treated with or without 3.5 μM GA for 24 h were evaluated. After treatment for 48 h, cells were detached by trypsin and resuspended in serum-free medium. Medium containing 10% FBS was applied to the lower chamber as a chemoattractant, and then cells were seeded on the upper chamber with 8 μm pore polycarbonate filters at a density of 1×10^6 cells/well in 50 μL of serum-free medium. The chamber was incubated for 6 h at 37 °C. At the end of the incubation, the cells that migrated to the lower surface of the membrane were fixed with methanol and stained with 5% Giemsa solution, and the cells on the upper surface of the membrane were carefully removed with a cotton swab. The migrating cells on the lower surface of the membrane filter were counted with a light microscope.

Visualization of the F-actin cytoskeleton. AGS cells were transfected with negative control siRNA or RhoB siRNA and then treated with or

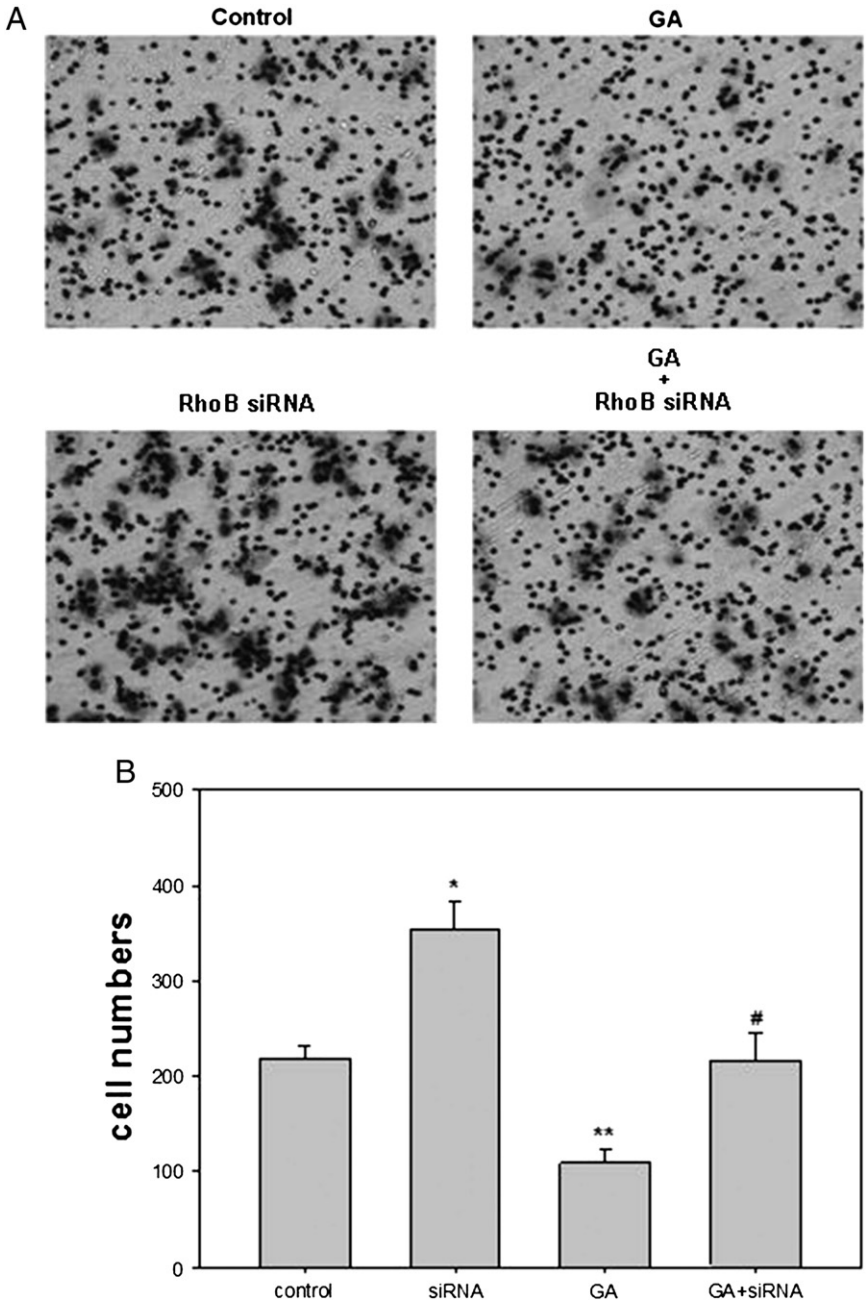

Fig. 3. Effects of gallic acid (GA) on cell migration of AGS cells assessed by Boyden chamber assay. AGS cells were transfected with control siRNA or RhoB siRNA and then treated with or without 3.5 μM GA for 24 h. Cells were plated in the upper chamber of a modified Boyden chamber containing a membrane, and the number of cells on the underside of the membrane was assessed 6 h later under light microscopy. (A) Representative photomicrographs of the membrane-associated cells were assayed by Giemsa stain. (B) Quantitative assessment of the mean number of cells. The data represent as mean ± SD from three independent experiments for each group. (*$p<0.0005$, **$p<0.00005$ compared with control, #$p<0.0005$ compared with GA).

without 3.5 μM GA for 24 h. AGS cells were seeded in 6-well plates at a density of 5×10^4 cells/well and cultured in DMEM containing 10% FBS. After 24 h, the medium was replaced by a serum-free medium for another 24 h. Tetramethylrhodamine B isothiocyanate (TRITC)-conjugated phalloidin (Sigma, St Louis, MO) was used to visualize polymerized F-actin microfilaments by fluorescence microscopy. In addition, 1 mg/mL of DAPI was added to visualize the nuclei. All images were taken using a Nikon upright fluorescence microscope (×100 objective). The AGS cells were then fixed in 3.7% formaldehyde in PBS for 10 min, permeabilized in 0.2% Triton X-100/PBS for 5 min, and stained with 50 μg/mL TRITC-conjugated phalloidin (Sigma) in PBS. After extensive washing with PBS, cells were briefly stained with DAPI (Sigma) to visualize nuclei.

Analysis of MMP-2/MMP-9 activity (gelatin zymography). AGS cells were transfected with control siRNA or RhoB siRNA then treated with or without 3.5 μM GA for 24 h. The serum-free medium of AGS cells after being treated by GA were the samples. The samples were subsequently transferred to gelatin zymography to analyze the activity of MMP-9 and MMP-2. The activities of MMP-2 and MMP-9 were assayed by gelatin zymography as described previously. Conditioned media were mixed with 1/5 volume of 312 mM Tris–HCl, pH 6.8, containing 10% SDS

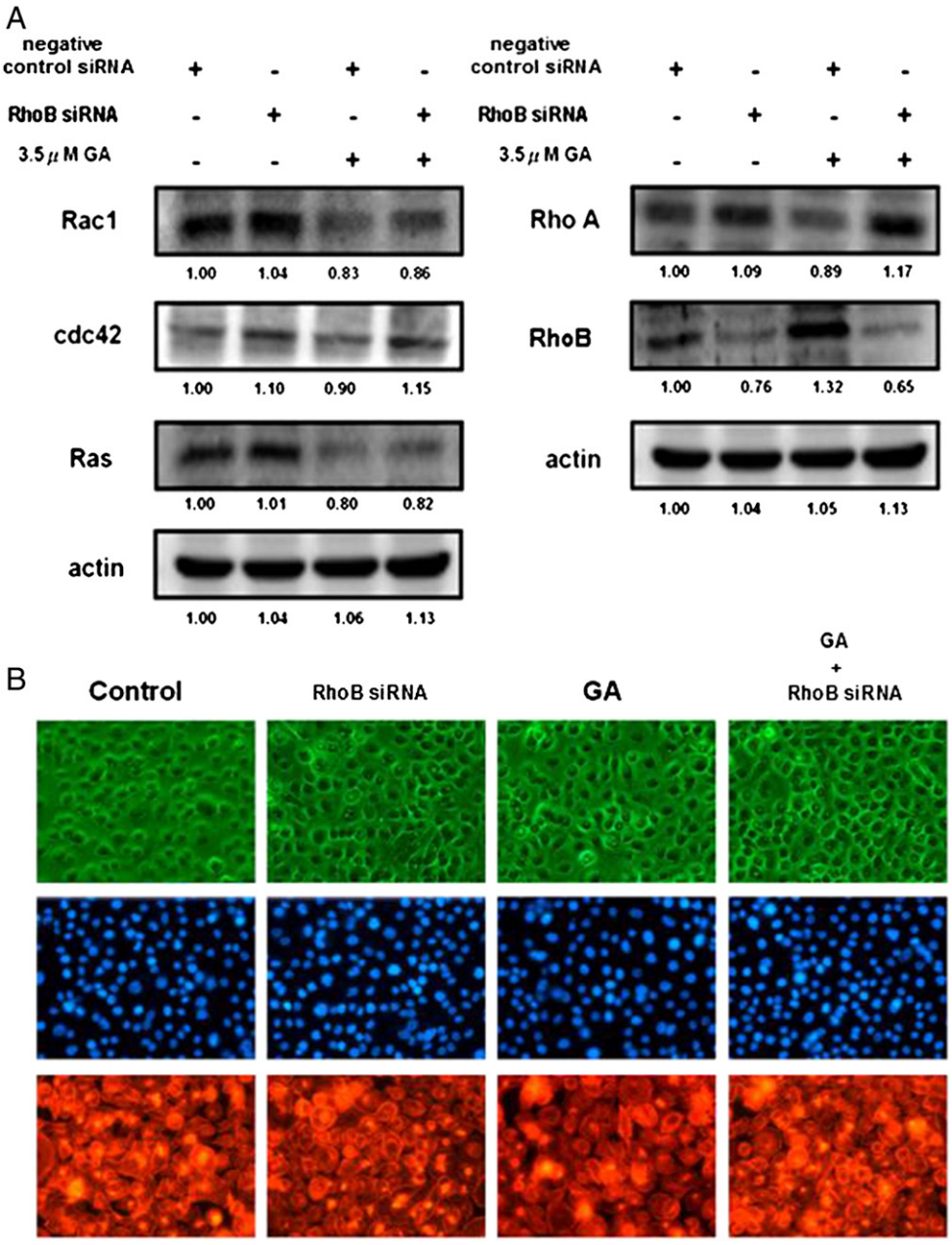

Fig. 4. (A) Immunoblot analysis of the expression of small GTPase family in AGS cells treated with gallic acid (GA).(A) AGS cells were transfected control siRNA or RhoB siRNA then treated with or without 3.5 μM GA for 24 h. The protein levels of Ras, cdc42, Rac1, RhoA, and RhoB were analyzed by Western blotting. β-actin was used for equal loading. The protein levels of Rac1, cdc42, Ras and RhoA were decreased when AGS cells were transfected with control siRNA and then treated with 3.5 μM GA. However, the protein level of RhoB was increased. On the contrary, the protein level of RhoB was decreased when AGS cells were transfected with RhoB siRNA and then treated with 3.5 μM GA. Values were the average of triplicate experiments. (B) Cytoskeletal F-actin patterns with phalloidin-TRITC in AGS cells treated with gallic acid (GA). AGS cells were transfected with control siRNA or RhoB siRNA and then treated with or without 3.5 μM GA for 24 h. Cells were fixed and labeled with phalloidin-TRITC for F-actin (red) and with the nucleic acid DAPI for DNA (blue). The results showed that F-actin patterns decreased and AGS cell became enlarged after treated with GA. However, this effect was eliminated when AGS cells were transfected with RhoB siRNA.

and 0.1% bromphenol blue, and loaded on 7.5% SDS-polyacrylamide gels that had been copolymerized with 0.1% gelatin (Sigma). Electrophoresis was performed under non-reducing conditions. Gels were washed twice for 60 min in wash buffer (50 mM Tris–HCl, pH 7.5, containing 1% Triton X-100) to remove SDS and then incubated in reaction buffer (50 mM Tris–HCl, pH 7.5, containing 1% Triton X-100, 10 mM $CaCl_2$, 150 mM NaCl, and 0.01% NaN_3) at 37 °C. Gels were stained with 0.2% Coomassie blue in 30% methanol/10% acetic acid for 60 min and destained in 30% methanol/10% acetic acid.

Experimental metastasis assay in nude mice. Five-week-old BALB/cAnN-Foxn1nu/CrlN male nude mice, weighing 20–30 g, were used in the experimental metastasis assay. AGS cells (1×10^6) were injected subcutaneously into the flanks of mice on day-0. GA was added into distilled water forming 0.25% and 0.5% solution for further experiments. The nude mice were divided into 5 groups. In group 1, matrix gel was injected only as control. In group 2, AGS tumor cells were injected, but no GA solution feeding was performed. In group 3, AGS tumor cells were injected and fed with 0.25% GA solution from day-3.

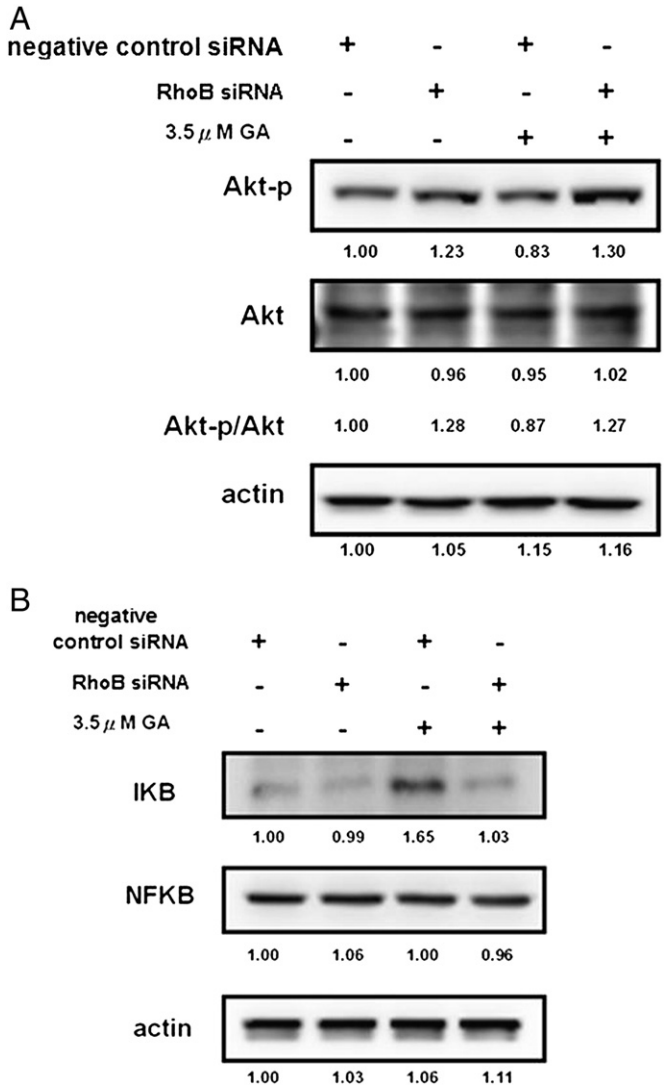

Fig. 5. (A) Immunoblot analysis of the expression of PI3K and AKT in AGS cells treated with gallic acid (GA). AGS cells were transfected with control siRNA or RhoB siRNA and then treated with or without 3.5 μM GA for 24 h. The protein levels of PI3K, AKT-1, and P-AKT were analyzed by Western blotting. There was a decrease in protein level of Akt-p and Akt-p/Akt ratio when AGS cells were transfected with control siRNA and then treated with 3.5 μM GA. However, this effect was abolished when AGS cells were transfected with RhoB siRNA and then treated with 3.5 μM GA. β-actin was used for equal loading. Values were the average of triplicate experiments. (B) Immunoblot analysis of the expression of IκB and NF-κB in AGS cells treated with gallic acid (GA). AGS cells were transfected control siRNA or RhoB siRNA then treated with or without 3.5 μM GA for 24 h. The protein levels of IκB and NF-κB were analyzed by Western blotting. There was an increase in protein level of IκB when AGS cells were transfected with control siRNA and then treated with 3.5 μM GA. However, this effect was eliminated when AGS cells were transfected with RhoB siRNA and then treated with 3.5 μM GA. β-actin was used for equal loading. Values were the average of triplicate experiments.

In group 4, AGS tumor cells were injected and fed with 0.5% GA solution from day-3. In group 5, matrix gel was injected and fed with 0.5% GA solution from day-3. The mice were sacrificed after 4 weeks. The sizes and weights of tumors were assessed. In addition, the tumor metastasis assay was measured by tumor sections stained with Hematoxylin and Eosin stain and immunohistochemical stain for RhoB.

Statistical analysis. Data are shown as mean ± standard deviation of three independent experiments and were evaluated by one-way analysis of variance (ANOVA). Significant differences were established at $p<0.05$.

Results

Effects of GA in expression of Rho B in AGS cells

Immunoblot analysis of the expression of RhoB was performed after AGS cells were treated with GA. AGS cells were treated with or without 3.5 μM GA and then harvested at different times (0, 3, 6, 12, 24, 36, 48 h). The protein levels of RhoB were analyzed by Western blotting. Increased expression of protein levels of RhoB was observed throughout the experimental period (3 to 48 h) (Fig. 1A). Especially we observed the obviously in the creation of RhoB at 24 to 36 h. Effects of GA on RhoB mRNA level in AGS cell were further analyzed. AGS cells were transfected with control siRNA or RhoB siRNA then treated with or without 3.5 μM GA for 24 h. Total RNA was isolated, and the mRNA expression was analyzed by RT-PCR. The results showed inhibition of RhoB mRNA when AGS cells were transfected with RhoB siRNA (Fig. 1B).

Effects of GA on AGS cells migration

Monolayers of growth-arrested AGS cells were transfected with control siRNA or RhoB siRNA then treated with or without 3.5 μM GA. Cells were scraped and the number of cells in the denuded zone (i.e., wound) was assessed at 0 and 24 h under light microscopy (Fig. 2A). Mean number of cells at 0 and 24 h in the denuded zone represents the average of three independent experiments (mean ± SD) (Fig. 2B). Migration of AGS cells was inhibited when treated with GA. However, this effect was abolished when AGS cells were transfected with RhoB siRNA. Effects of GA on cell migration of AGS cells were further assessed by Boyden chamber assay. AGS cells were transfected with control siRNA or RhoB siRNA and then treated with or without 3.5 μM GA for 24 h. Cells were plated in the upper chamber of a modified Boyden chamber containing a membrane, and the number of cells on the underside of the membrane was assessed 6 h later under light microscopy (Fig. 3A). Migration of AGS cells was inhibited when treated with GA. Once again, this effect was eliminated when AGS cells were transfected with RhoB siRNA (Fig. 3B).

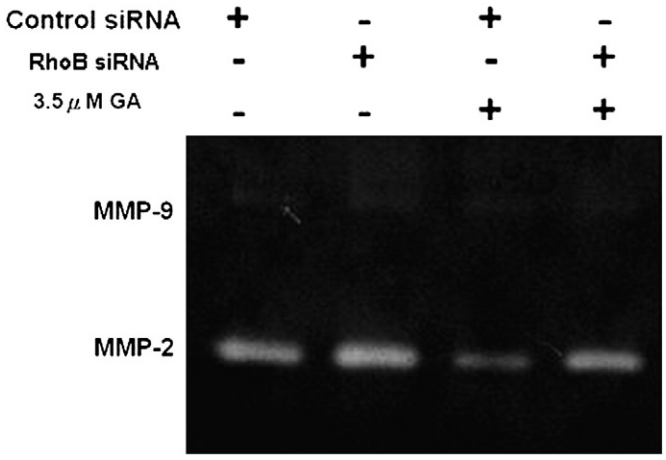

Fig. 6. Effects of gallic acid (GA) on MMP-9 and MMP-2 activity. AGS cells were transfected with control siRNA or RhoB siRNA then treated with or without 3.5 μM GA for 24 h. The serum-free medium of AGS cells after being treated by GA was the samples. The samples were subsequently transferred to gelatin zymography to analyze the activity of MMP-9 and MMP-2. There was a decrease in protein level of MMP-9 and MMP-2 when AGS cells were transfected with control siRNA and then treated with 3.5 μM GA (Fig. 6). However, this effect was abolished when AGS cells were transfected with RhoB siRNA and then treated with 3.5 μM GA.

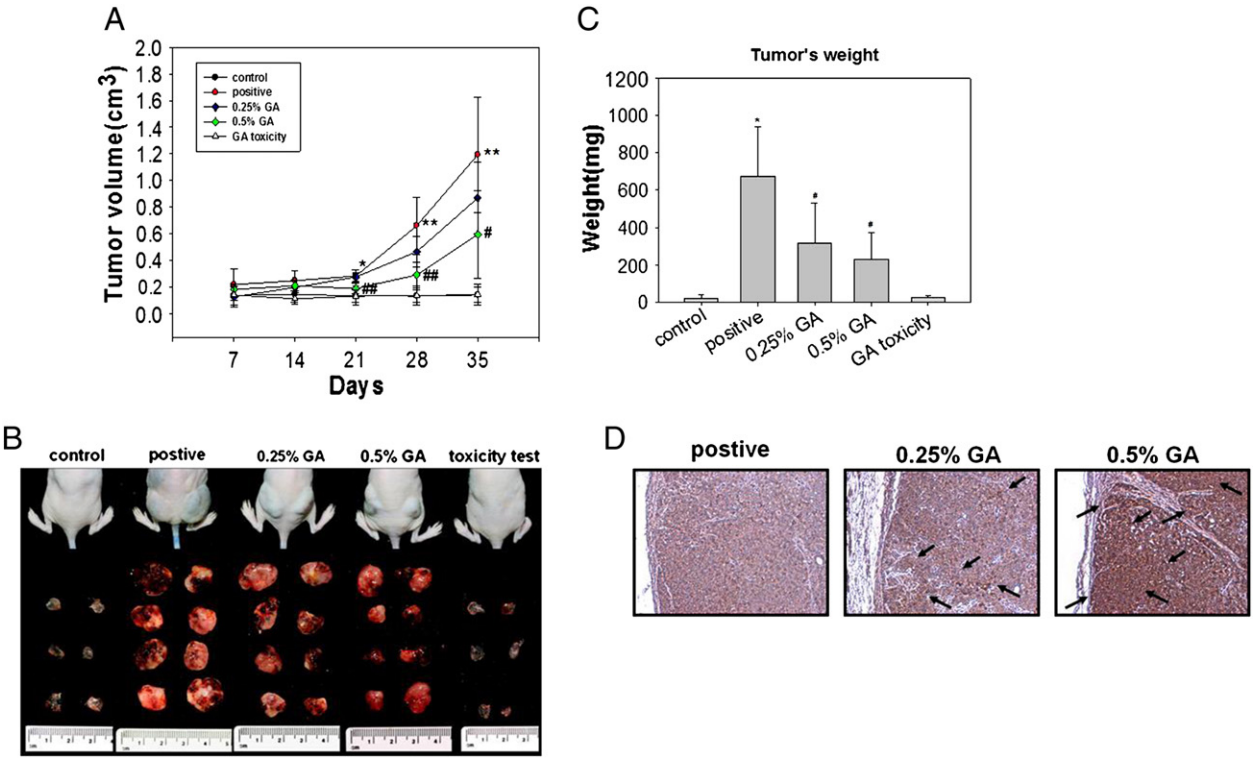

Fig. 7. Gallic acid (GA) inhibits tumor growth in nude mice model. AGS cell were resuspended in serum-free medium at 1×10^7 cells/mL and aliquot of the cells (10 μL) were mixed with 10 μL Matrigel Matrix. AGS cell with Matrigel Matrix was injected subcutaneously into both thighs of mice to initiate tumor growth for 7 days. The mice were then treated without or with two different concentrations GA (0.25%, 0.5%) for 1 month. The nude mice were sacrificed after 1 month. Tumor size was measured weekly in two dimensions throughout the study (A) (* $p<0.005$ and ** $p<0.001$, positive group compared with control group; # $p<0.05$ and ## $p<0.01$, positive group compared with 0.5% GA group). The typical appearance of tumors in each group was demonstrated (B). The tumor's weight for each group was expressed as mean ± SD from three independent experiments (C) (* $p<0.0005$, compared with control group; # $p<0.05$ and # # $p<0.001$ compared with GA toxicity group). Tumors were removed from mice at week 5 after AGS cell injection, and stained with IHC for Rho B (D). Arrow indicated positive staining for Rho B.

The expression of small GTPase family in AGS cells treated with gallic acid (GA)

AGS cells were transfected with control siRNA or RhoB siRNA and then treated with or without 3.5 μM GA for 24 h. The protein levels of Rac1, cdc42, Ras, RhoA, and RhoB were analyzed by Western blotting. There was a decrease in protein levels of Rac1, cdc42, Ras and RhoA when AGS cells were transfected with control siRNA and then treated with 3.5 μM GA. However, the protein level of RhoB was increased (Fig. 4A). In contrast, the protein level of RhoB was decreased when AGS cells were transfected with RhoB siRNA and then treated with 3.5 μM GA.

The effect of GA on cytoskeletal F-actin patterns in AGS cells

AGS cells were transfected with control siRNA or RhoB siRNA and then treated with or without 3.5 μM GA for 24 h. Cells were fixed and labeled with phalloidin-TRITC for F-actin (red) and with the nucleic acid DAPI for DNA (blue). The results showed F-actin patterns decreased and AGS cell became enlarged after being treated with GA (Fig. 4B). However, this effect was eliminated when AGS cells were transfected with RhoB siRNA.

The effect of GA on the expression of Akt, IκB and NF-κB in AGS cells

In a recent study, they demonstrate that Ras downregulates RhoB expression by a phosphatidylinositol 3-kinase (PI3K)- and Akt- but not Mek-dependent mechanism. And they also demonstrate that ectopic expression of RhoB inhibits Ras, PI3K, and Akt induction of transformation and migration (Jiang et al., 2004). To further investigate the role of RhoB siRNA in GA induced AGS cells, the mechanisms of Akt and NF-κB signal were performed. First, as shown in Fig. 5A, only transfect RhoB siRNA could increase the level of Akt-p/Akt (1.28 fold) and treatment of GA (3.5 μM) and negative control siRNA decreased protein levels of Akt-p/Akt (0.87 fold). However, the Akt-p/Akt level was not decreased (1.27 fold) by co-treatment with RhoB siRNA and GA. But we did not observe an obvious change on P13K protein expression (Fig. 5A). Our recent study showed that GA could suppress NFκB transcription activity by increasing IκB protein expression. Furthermore, there was an increase in protein level of IκB when cells were treated with GA and negative siRNA. Treatment of siRNA for RhoB significantly eliminated the expression of IκB (Fig. 5B). In this data, we did not observe an obvious change on NFκB protein expression. In Fig. 5, we could find that GA suppressed Akt and NFκB activation via inducing RhoB gene expression.

Effects of GA on MMP-9 and MMP-2 activity

AGS cells were transfected with control siRNA or RhoB siRNA then treated with or without 3.5 μM GA for 24 h. The serum-free medium of AGS cells after being treated by GA were the samples. The samples were subsequently transferred to gelatin zymography to analyze the activity of MMP-9 and MMP-2. We could clearly observed AGS cells secreted MMP-2. There was a decrease in protein level of MMP-2 when AGS cells were transfected with control siRNA and then treated with 3.5 μM GA (Fig. 6). However, this effect was abolished when AGS cells were transfected with RhoB siRNA and then treated with 3.5 μM GA.

Effects of GA inhibit tumor growth in nude mice model

AGS cells with Matrigel Matrix were injected subcutaneously into both thighs of mice to initiate tumor growth for 7 days. The mice

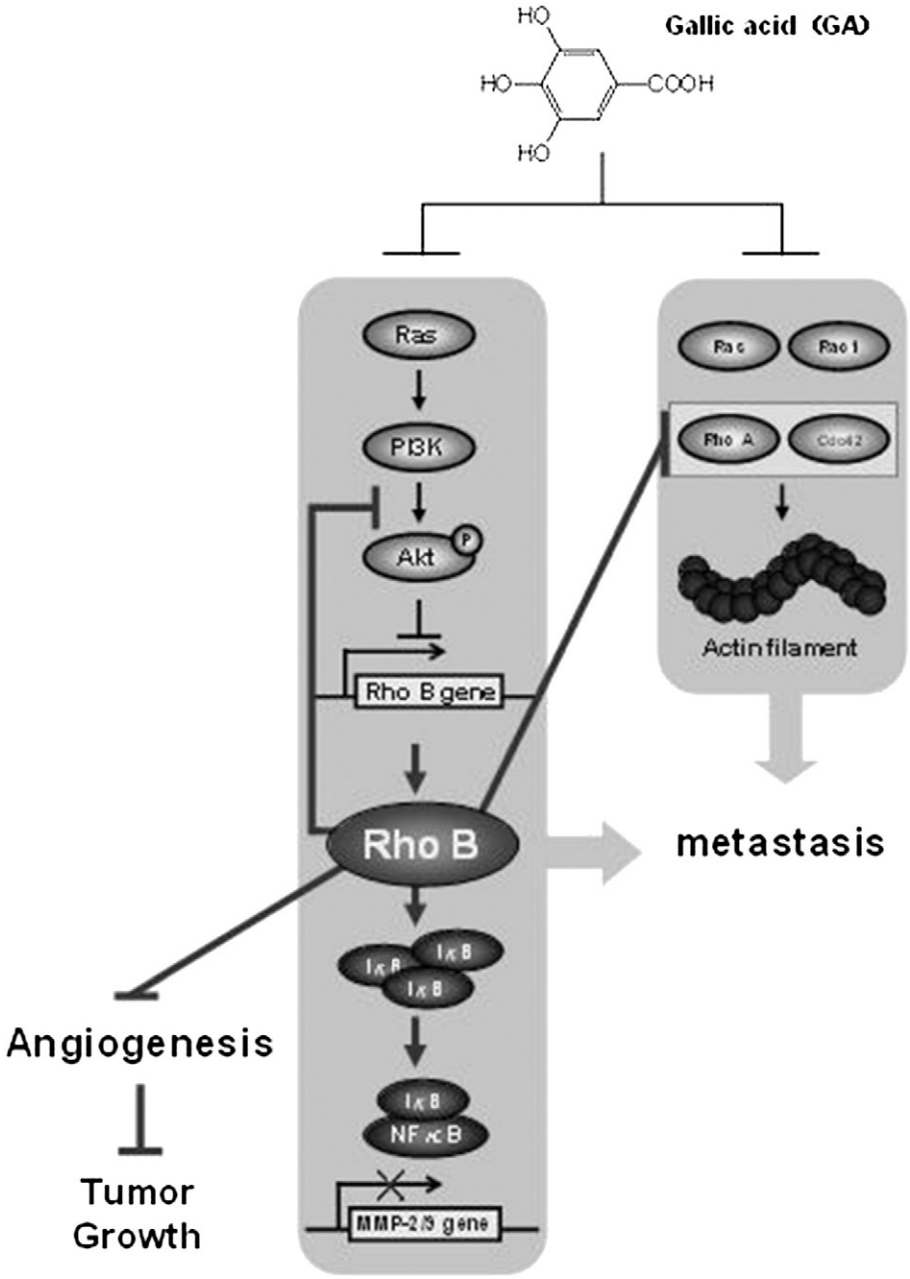

Fig. 8. The probable pathway for gallic acid inhibition of AGS metastasis involving Rho B.

were then treated with or without three different concentrations of GA (0.25%, 0.5% and toxicity) for 1 month. Tumor sizes were measured weekly in two dimensions throughout the study. The tumor weights of mice treated with GA (0.5%) were significantly reduced as compared with matrix gel control, suggesting that the higher dose of GA was able to enhance tumor regression (Figs. 7A–C). Expression of RhoB was observed using immunohistochemical stain in the tumors of nude mice fed with GA solution (Fig. 7D). However, the dosages (0.25% and 0.5%) of GA treated in nude mice have shown obviously anticancer effect.

Discussion

The findings of the present study revealed that GA inhibits metastasis and invasive growth of gastric cancer cells via increased expression of RhoB, downregulation of AKT/small GTPase signals and inhibition of NF-κB activity. Thus, GA might be a potential agent in treating gastric cancer (Fig. 8).

Although they share more than 85% sequence identity, RhoA, RhoB and RhoC appear to play distinctive roles in carcinogenesis. RhoA has been shown to be overexpressed in a series of malignancies including breast cancer, colon cancer, ovarian cancer and lung cancer (Fritz et al., 1998; Horiuchi et al., 2003; Kamai et al., 2001, 2003). RhoC has been revealed as a key regulator in migration and metastasis of melanoma (Boone et al., 2009). In contrast, the role of RhoB in human cancer is equivocal. Some studies suggest that RhoB acts as a suppressor or negative modifier in cancer as evidenced by the downregulation of expression of RhoB protein in head and neck carcinoma and lung cancer (Adnane et al., 2002; Mazieres et al., 2004; Sato et al., 2007). Loss of RhoB was found to be associated with an increased susceptibility to

chemical carcinogenesis in mice (Liu et al., 2001). RhoB expression is reduced in several tumor types compared with non-cancer tissues and can contribute to tumor growth by regulating apoptosis (Huang and Prendergast, 2006). Its tumor suppressor activity could also reflect a role in inhibiting cancer cell migration. In our study, GA could increase RhoB expression in AGS cells. Increased expression of protein levels of RhoB was observed throughout the experiment period (6 to 36 h) (Fig. 1A). In addition, there was an inhibition of Rho B mRNA when AGS cells were transfected with RhoB siRNA. These results indicate that GA enhanced the expression of RhoB mRNA and subsequently resulted in increased expression of the protein level of RhoB.

With the same model of AGS cells transfected with RhoB siRNA, migration of AGS cells was inhibited when treated with GA. However, this effect was abolished when AGS cells were transfected with RhoB siRNA. Effects of GA on cell migration of AGS cells were further assessed by Boyden chamber assay. Migration of AGS cells was inhibited when treated with GA. Once again, this effect was eliminated when AGS cells were transfected with RhoB siRNA. These results indicate that increased expression of RhoB induced by GA might lead to the inhibition of AGS cell migration.

The protein levels of Rac1, cdc42, Ras, RhoA, and RhoB were analyzed by Western blotting. There was a decrease in protein levels of Rac1, cdc42, Ras and RhoA when AGS cells were transfected with control siRNA and then treated with 3.5 μM GA. However, the protein level of Rho B was increased. In contrast, the protein level of RhoB was decreased when AGS cells were transfected with RhoB siRNA and then treated with 3.5 μM GA. Ras-homologous (Rho) GTPases play a pivotal role in the regulation of numerous cellular functions associated with malignant transformation and metastasis. Members of the Rho family of small GTPases are key regulators of actin reorganization, cell motility, cell–cell and ECM adhesion as well as of cell cycle progression, gene expression, and apoptosis (Chen et al., 2000). RhoB shares 86% amino acid sequence identity with RhoA, yet the roles of the low-molecular-weight GDP/GTP binding GTPases in oncogenesis are quite different. While RhoA, like other GTPase family members such as Ras, Rac1, and cdc42, promotes oncogenesis, invasion, and metastasis (Khosravi-Far et al., 1995), emerging evidence points to a tumor-suppressive role for RhoB (Chen et al., 2000). Furthermore, PI3K is an effector of Ras function and has been shown to be required for both the development and maintenance of tumors driven by mutant H-Ras (Lim et al., 2005). Based on the finding that there was decreased expression of Ras, cdc42, Rac1 and RhoA, further studies were performed to evaluate the effects of GA on the skeletal structure of the AGS cells. The results showed F-actin patterns decreased and AGS cell became enlarged after being treated with GA. Furthermore, this effect was eliminated when AGS cells were transfected with RhoB siRNA.

The activation of Akt and NF-κB was analyzed after AGS cells were transfected with control siRNA or RhoB siRNA and then treated with or without GA. There was a decrease in protein level of Akt-p/Akt ratio when AGS cells were transfected with control siRNA and then treated with GA. However, this effect was abolished when AGS cells were transfected with RhoB siRNA and then treated with GA. Furthermore, there was an increase in protein level of IκB when AGS cells were transfected with control siRNA and then treated with GA. However, this effect was eliminated when AGS cells were transfected with RhoB siRNA and then treated with GA. NF-κB is a multi-subunit transcription factor involved in cellular responses to viral infection and inflammation. NF-κB is maintained in the cytoplasm through interactions with an inhibitor of NF-κB (IκB). Upon dissociation, NF-κB moves into the nucleus and promotes cancer cell proliferation, angiogenesis, and metastasis. Several studies have documented that the quantity of IκB could control NF-κB nuclear translocation and consequently influence the expression of MMP-2 in several types of human cells (Javelaud et al., 2002; Park et al., 2007). In this study, there was an increase in IκB when AGS cells were treated with GA. However, the protein expression of NF-κB was not changed under the same condition. This result suggests that an increase of cytoplasmic IκB could bind with NF-κB thereby inhibiting NF-κB activity. In addition, the PI3K signal transduction pathway regulates cell metastasis of melanoma and is closely associated with the development and progress of various tumors (Hennessy et al., 2005). In this study, the protein levels of Akt-p decreased when treated with GA. However, this effect was eliminated when AGS cells were transfected with RhoB siRNA. In addition, GA might increase IκB binding to NF-κB and inhibited the NF-κB transcription activity, then suppress the PI3K/AKT pathway, subsequently leading to the anti-metastasis of AGS cell.

The effects of GA on MMP-9 and MMP-2 activity were analyzed after AGS cells were transfected with control siRNA or RhoB siRNA then treated with or without GA. There was a decrease in protein level of MMP-2 when AGS cells were transfected with control siRNA and then treated with 3.5 μM GA. However, this effect was abolished when AGS cells were transfected with RhoB siRNA and then treated with GA. Many studies of tumor invasion and metastases have supported the postulation that degradation of the extracellular MMPs plays a central role (Libra et al., 2009). Two of these enzymes, MMP-2 and MMP-9, correlate with the processes of tumor cell invasion and metastasis in human cancers (Libra et al., 2009). Additionally, MMP gene expression is chiefly regulated by transcriptional factors (for example, NF-κB and AP-1) via the PI3K/AKT pathway (Chung et al., 2004; Fritz and Kaina, 2006).

In a recent study, they found that RhoA promoted the gastric cancer cell proliferation and RhoC stimulated migration and invasion of the gastric cancer cell. RhoB expression, however, significantly inhibited the proliferation, migration and invasion of the gastric cancer cells and also enhanced the chemosensitivity of these cells to anticancer drugs. It appears that RhoB plays an opposing role from that of RhoA and/or RhoC in gastric cancer cells (Zhou et al., 2011). In the nude mice model, we indeed observed RhoB overexpression in the tumors which was fed with GA solution (Fig. 7D). In addition, RhoB overexpression tumors were smaller than the positive group (Figs. 7A and B). Our work suggests a negative role of RhoB in gastric cancer progression and GA might be a potential agent for gastric cancer therapy.

Conflict of interest

The authors declare that there are no conflicts of interest.

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