

Original Article

# Gambogic acid inhibits TNF- $\alpha$ -induced invasion of human prostate cancer PC3 cells *in vitro* through PI3K/Akt and NF- $\kappa$ B signaling pathways

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**Aim:** To investigate the mechanisms underlying the inhibitory effect of gambogic acid (GA) on TNF- $\alpha$ -induced metastasis of human prostate cancer PC3 cells *in vitro*.

**Methods:** TNF- $\alpha$ -mediated migration and invasion of PC3 cells was examined using migration and invasion assays, respectively. NF- $\kappa$ B transcriptional activity and nuclear translocation were analyzed with luciferase reporter gene assays, immunofluorescence assays and Western blots. The ability of p65 to bind the promoter of Snail, an important mesenchymal molecular marker, was detected using a chromatin immunoprecipitation (ChIP) assay. After treatment with Snail-specific siRNA, the expression of invasiveness-associated genes was measured using quantitative real-time PCR and Western blot.

**Results:** GA significantly inhibited the viability of PC3 cells at 1–5  $\mu$ mol/L, but did not produce cytotoxic effect at the concentrations below 0.5  $\mu$ mol/L. GA (0.125–0.5  $\mu$ mol/L) dose-dependently inhibited the migration and invasion of PC3 cells induced by TNF- $\alpha$  (10 ng/mL). Moreover, the TNF- $\alpha$ -mediated activation of phosphatidylinositol-3-OH kinase/protein kinase B (PI3K/Akt) and NF- $\kappa$ B pathways was suppressed by GA (0.5  $\mu$ mol/L). Furthermore, this anti-invasion effect of GA was associated with regulation of Snail. Snail expression was significantly down-regulated by treatment with GA (0.5  $\mu$ mol/L) in the TNF- $\alpha$ -stimulated PC3 cells.

**Conclusion:** GA inhibits TNF- $\alpha$ -induced invasion of PC3 cells via inactivation of the PI3K/Akt and NF- $\kappa$ B signaling pathways, which may offer a novel approach for the treatment of human prostate cancer.

**Keywords:** gambogic acid; human prostate cancer; tumor metastasis; tumor invasion; tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ); PI3K/Akt signaling pathway; NF- $\kappa$ B signaling pathway; Snail

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## Introduction

TNF- $\alpha$  is a potent pro-inflammatory cytokine involved in the regulation of multiple physiological and pathological processes, including inflammatory reaction, immunity, cachexia and tumor progression<sup>[1]</sup>. As a multifunctional cytokine, TNF- $\alpha$  is a double-edged sword in the treatment of malignant disease. High doses of human recombinant TNF- $\alpha$  were found to exert a powerful anti-cancer effect by inducing cell apoptosis and inhibiting tumor blood vessel formation. However, in low doses and with chronic stimulation, TNF- $\alpha$  could induce angiogenesis, proliferation, invasion and metastasis of tumor cells<sup>[2]</sup>.

As an endogenous tumor-promoting factor TNF- $\alpha$  plays an important role in promoting invasion in many cancer types

including prostate cancer. Some crosstalk between pathways that are activated by TNF- $\alpha$ , such as NF- $\kappa$ B and PI3K/Akt<sup>[3,4]</sup>, is correlated with TNF- $\alpha$ -mediated tumor cell migration and invasion. For example, sustained activation of NF- $\kappa$ B by TNF- $\alpha$  has been reported to be involved in the regulation of tumor cell invasion via up-regulated expression of macrophage migration inhibitory factor (MIF), enhanced matrix metalloproteinases (MMPs) production and stabilized Snail<sup>[5,6]</sup>. In addition, TNF- $\alpha$  also induces a cell-invasion signal through the PI3K/Akt pathway. Activation of Akt by TNF- $\alpha$  could enhance the transcription of Zeb2 and suppress the expression of E-cadherin<sup>[7]</sup>. Considering the critical role of TNF- $\alpha$  in tumor invasion, inhibiting the TNF- $\alpha$  signaling pathway may provide an important base for new targeting therapies in the intervention of human prostate cancer.

Gambogic acid (GA) is a compound extracted from natural resin gamboges, which has been widely used in traditional Chinese medicine<sup>[8]</sup>. It has been shown that GA exhibited anti-

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proliferative and pro-apoptotic effects on breast, oral, stomach and liver cancer cells<sup>[9–13]</sup>. Although it is clear that GA inhibited the growth of different tumor cell lines by promoting apoptosis and repressing proliferation, the relationship between GA and tumor metastasis remains unclear.

The aim of this study is to evaluate whether GA can inhibit the TNF- $\alpha$ -mediated invasion in human prostate cancer PC3 cells and to further explore its functional mechanism.

## Materials and methods

### Cell culture

Human prostate cancer PC3 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin. The cells were incubated in 5% CO<sub>2</sub> at 37 °C.

### Antibodies and reagents

GA was purchased from Sigma-Aldrich (St Louis, MO, USA), dissolved in DMSO at a concentration of 25 mmol/L, aliquoted and stored at -20 °C. Recombinant TNF- $\alpha$  was obtained from R&D systems (Minneapolis, MN, USA). Polyclonal antibodies directed against MMP-9, fibronectin and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Snail, anti-E-cadherin and anti-NF- $\kappa$ B p65 were purchased from Abcam (Abcam, USA). Anti-Akt, anti-phosphor-Akt (Ser<sup>473</sup>), anti-GSK-3 $\beta$  and anti-phosphor-GSK-3 $\beta$  (Ser<sup>9</sup>) were obtained from Cell Signaling Technology (Beverly, MA, USA). PI3K inhibitor LY294002 (5  $\mu$ mol/L) was purchased from Sigma-Aldrich (St Louis, MO, USA).

### Cell viability assay

The impact of GA on cell viability was assessed by the MTT assay (Beyotime Institute Biotechnology, China). Briefly, PC3 cells at 5 $\times$ 10<sup>3</sup> per well were seeded in 96-well plates and treated with different concentrations of GA for 24 h. The relative level of cell viability in each group of cells compared to control unmanipulated cells was calculated, and the unmanipulated control cells were designated as 100%.

### Migration and invasion assays

Cell invasion assays were performed in a Boyden chemotaxis chamber (matrigel-coated polycarbonate membrane, Costar, USA) according to the manufacturer's protocol. Briefly, PC3 cells (5 $\times$ 10<sup>4</sup>) were suspended in serum-free RPMI-1640 medium and were placed into the upper chamber in the presence of various concentrations of GA. In the lower chamber, serum-free RPMI-1640 containing 10 ng/mL TNF- $\alpha$  served as a chemoattractant. After incubation for 24 h, the cells in the upper chamber were carefully wiped with cotton swabs, and the cells at the bottom of the membrane were fixed and stained with 0.1% crystal violet. Then, the stained cells that invaded the lower surface of the membrane were counted under the microscope. Migration assays were carried out using the same procedure, except that the polycarbonate membrane was not coated with matrigel, and the incubation time was 12 h.

### Western blot analysis

Briefly, cells were harvested and lysed with RIPA buffer containing protease inhibitor (Pierce, USA). After quantification of the protein concentrations using the BCA protein assay reagent kit (Pierce, USA), an equal amount of proteins (30  $\mu$ g) was resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. Subsequently, the membranes were blocked with 5% skim milk and incubated with the antibodies as described above. Protein bands were visualized using the super signal kit, according to the manufacturer's instructions (Pierce, USA).

### Immunofluorescence assay

The cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.5% Triton X-100 for 10 min at room temperature. Then, the cells were washed with PBS for three times, blocked with 10% goat serum for 1 h at room temperature and incubated with primary anti-NF- $\kappa$ B p65 antibody overnight at 4 °C. Then, the cells were incubated with rabbit IgG antibody labeled with Cy3 and viewed under a fluorescence microscope.

### Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using the TRIzol Reagent kit (Invitrogen, USA) and reverse transcription was performed according to the manufacturer's protocol. The relative levels of target gene mRNA transcripts to that of the control were determined by quantitative real-time polymerase chain reaction (qRT-PCR), using cDNA as a template, the specific primers and the SYBR premix system. The sequences of the specific primers used in the present study were as followed: Snail (NM\_005985), forward primer 5'-CTTCCAGCAGC-CCTACGA-3', reverse primer 5'-AGCCTTCCCACTGTC-CTC-3'; MMP-9 (NM\_004994), forward primer 5'-CGCTGGCTTAGATCATTC-3', reverse primer 5'-CAGGGCGAG-GACCATAGA-3'; E-cadherin (NM\_004360), forward primer 5'-ATCTTCAATCCCACCACG-3', reverse primer 5'-TGTA-GAATGTACTGCTGCTT-3'; fibronectin (NM\_212482), forward primer 5'-ATGGAGGAAGCCGAGGTT-3', reverse primer 5'-AGCGGTTTTCGATGGTAC-3'; and GAPDH (NM\_002046) forward primer 5'-TGAAGGTCGGAGTCAACG G-3', reverse primer 5'-CCTGGAAGATGGTATGGG-3'. The level of GAPDH mRNA transcript was used to normalize all reported gene expression levels, and the data were analyzed using the 2<sup>- $\Delta\Delta$ Ct</sup> method.

### Transfection and luciferase reporter gene assay

Briefly, PC3 cells at 1 $\times$ 10<sup>6</sup> cells per well were plated in 6-well plates and grown to approximately 70% confluence. Then, the cells were transiently co-transfected with pGL6-NF- $\kappa$ B-TA-luc (Beyotime Institute Biotechnology, China) and pRL-TK (Promega) for normalizing transfection efficiency. Six hours after transfection, the cells were pretreated with 10 ng/mL TNF- $\alpha$  for 12 h and subsequently incubated with or without GA for an additional 12 h, then harvested with passive lysis buffer

(Promega). The NF- $\kappa$ B transcriptional activity was assayed by using the dual luciferase system (Promega). The results were normalized to the luciferase activity of the internal control.

### Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using the ChIP Assay kit (Beyotime Institute Biotechnology, China) according to the manufacturer's protocol. To confirm the ability of p65 to bind to the Snail promoter, an anti-NF- $\kappa$ B p65-specific antibody was used to immunoprecipitate regions of the Snail promoter bound by anti-NF- $\kappa$ B p65 in this study. NF- $\kappa$ B p65-immunoprecipitated DNA was amplified with PCR using specific primers to analyze the NF- $\kappa$ B p65 binding site of the putative Snail promoter. The Snail promoter-specific primers were described as follows: forward primer 5'-GCCTCGCTTCGCTCGACGTC-3' and reverse primer 5'-AGGCCACTCCCCGAGCAGGT-3'.

### RNA interference

The mRNA sequence of Snail was obtained from GenBank (NM\_005985), and the targeting sequences were designed using an RNAi algorithm available online. The siRNA sequences for Snail as follows: Snail siRNA-1, 5'-GCCCUCGACCCCAAUCGGtt-3' (sense), 5'-CCGAUUGGGGUCG-GAGGGCtt-3' (antisense); Snail siRNA-2, 5'-UCGGAAGC-CUAACUACAGCtt-3' (sense), 5'-GCUGUAGUUAGGCUUC-CGAtt-3' (antisense); and Snail siRNA-3, 5'-UUGCCUG-GCCUCCCUUCGtt-3' (sense), 5'-CGAAGGGAGGCCAG-GCAAtt-3' (antisense). Snail-specific siRNAs and a nonsense siRNA (control siRNA) were obtained from Ribobio (Guangzhou, China). PC3 cells were transfected using Translipid reagent (TransGen, China) according to the manufacturer's protocol.

### Statistical analysis

The results are presented as the mean $\pm$ SD of at least three independent experiments. Statistical analysis was performed using one-way ANOVA or Student-Newman-Keuls (SNK)-

*q* Test. Differences were considered statistically significant when *P* values <0.05.

## Results

### Cytotoxicity of GA on PC3 cells

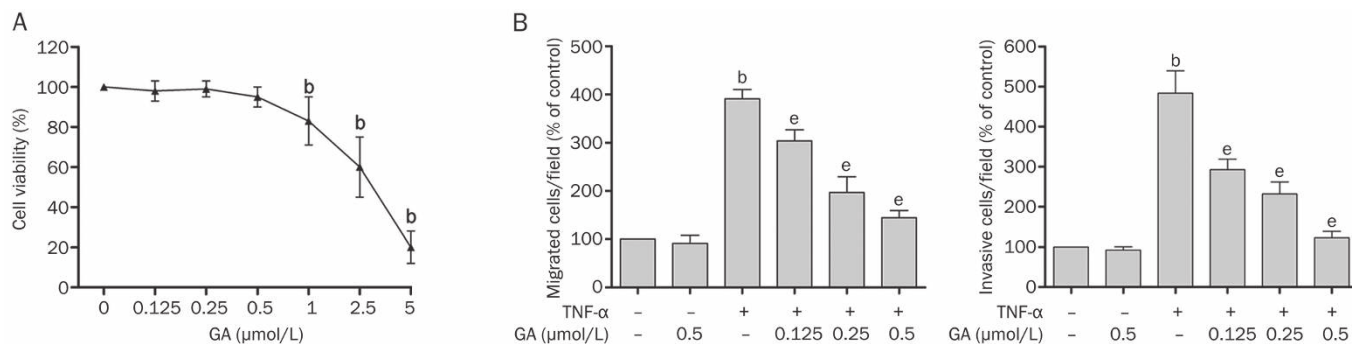
A series of concentrations of GA were tested on cell viability using an MTT assay. The results showed that GA with concentrations over 0.5  $\mu$ mol/L markedly inhibited cell viability of PC3 cells. However, low concentrations of GA (<0.5  $\mu$ mol/L) did not produce obvious cytotoxic effect on PC3 cells (Figure 1A). Therefore, a concentration of  $\sim$ 0.5  $\mu$ mol/L GA was chosen for the subsequent experiments.

### Inhibition of TNF- $\alpha$ -induced cell migration and invasion by GA

To investigate whether GA exerts a regulatory effect on cell migration and invasion, TNF- $\alpha$  (10 ng/mL) was applied to the lower chamber as a chemoattractive agent to induce *in vitro* migration and invasion of PC3 cells. Compared to the control, TNF- $\alpha$  could significantly induce migration and invasion of PC3 cells. Interestingly, administration of GA (0.5  $\mu$ mol/L) caused an inhibition of PC3 cell migration and invasion induced by TNF- $\alpha$ . Further studies found that GA inhibited TNF- $\alpha$ -induced cell migration and invasion in a dose-dependent manner (Figure 1B).

### Involvement of the PI3K/Akt signaling pathway in GA-induced inhibition of cell invasion by TNF- $\alpha$

Previous studies have reported that the PI3K/Akt/GSK-3 $\beta$  pathway was involved in cell migration and invasion<sup>[14, 15]</sup>. Akt and GSK-3 $\beta$  are both the critical components of signal transduction following PI3K activation<sup>[16]</sup>. We therefore asked whether the PI3K/Akt/GSK-3 $\beta$  pathway is associated with the TNF- $\alpha$ -induced cell invasion of PC3 cells. Following TNF- $\alpha$  treatment, PC3 cells showed a striking phosphorylation of Akt, whereas total Akt level remains unaltered. This increased phosphorylation of Akt reached its peak at 2 h after the TNF- $\alpha$  (10 ng/mL) application and returned back to the basal level

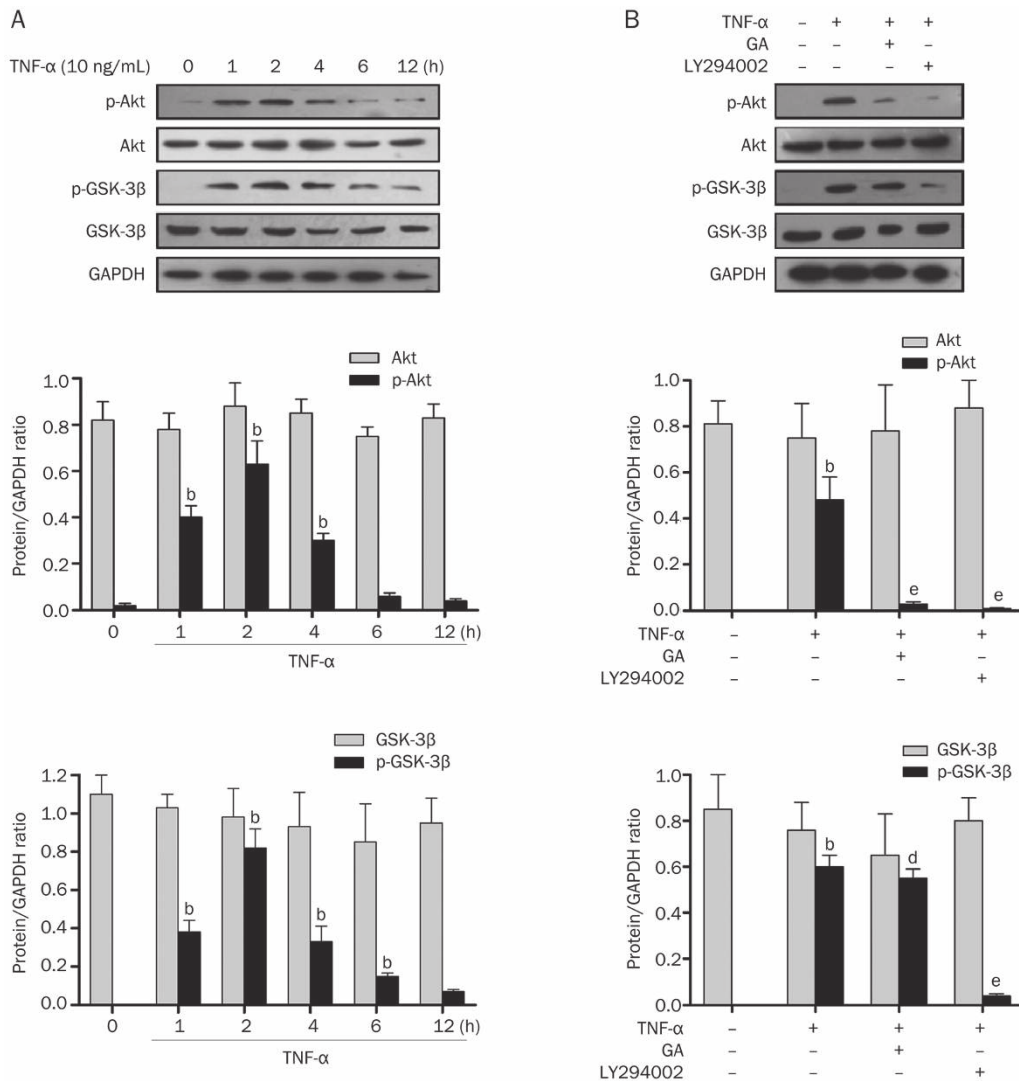


**Figure 1.** GA inhibits TNF- $\alpha$ -induced invasion of PC3 cells. (A) The effect of GA on the viability of PC3 cells. Cells were treated with GA at the indicated concentrations for 24 h. Viability of PC3 cells was determined by an MTT assay. (B) Inhibitory effect of GA on TNF- $\alpha$ -induced migration and invasion of PC3 cells. PC3 cells ( $5 \times 10^4$ ) were seeded into the upper compartment and treated with GA at the indicated concentrations. TNF- $\alpha$  (10 ng/mL) was applied to the lower chamber as a chemoattractive agent. Then, the cells were incubated for 12 h (migration assay) or 24 h (invasion assay). The migrating or invading cells were counted under the microscope. Data are presented as the mean $\pm$ SD of each group of cells from three independent experiments. <sup>b</sup>*P*<0.05 vs control group; <sup>a</sup>*P*<0.05 vs TNF- $\alpha$  alone treatment group. Imaged at 200 $\times$  magnification.

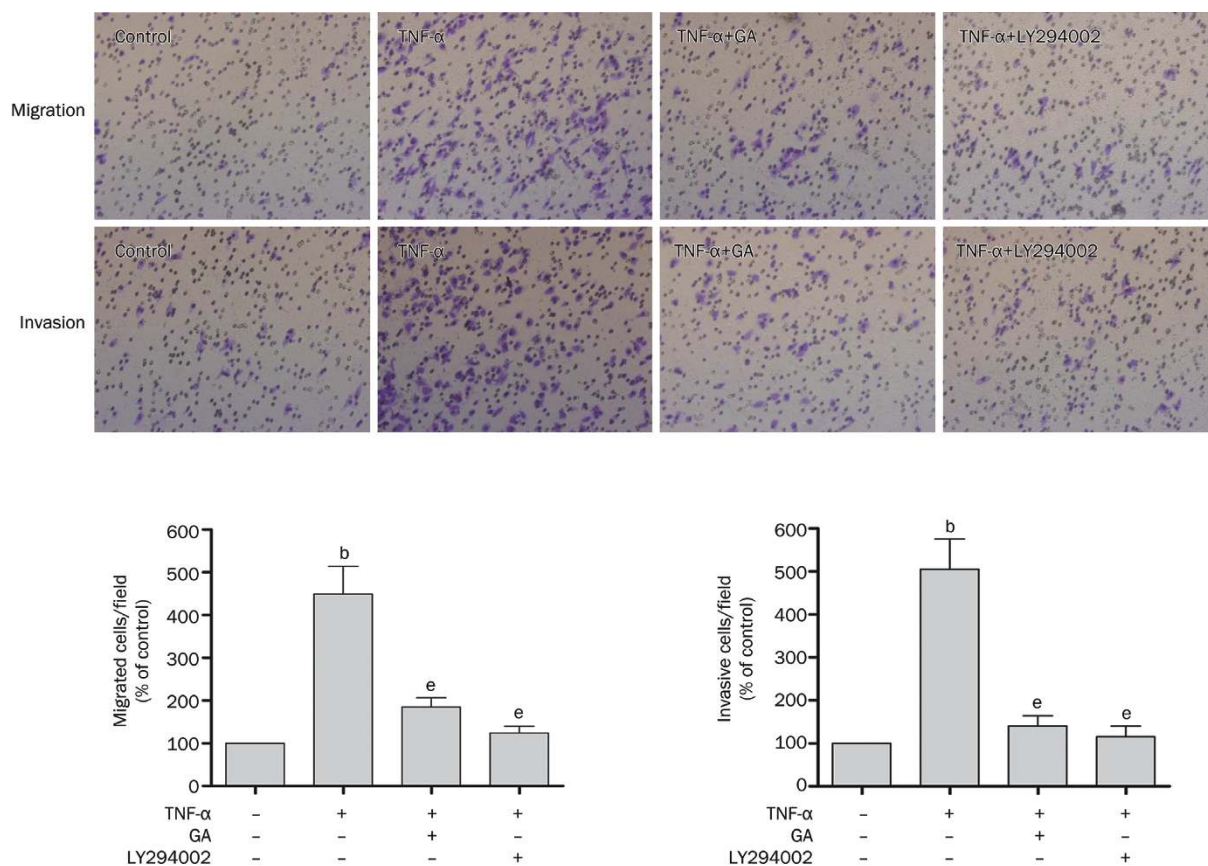
between 6–12 h after the application. In parallel, GSK-3 $\beta$  displayed dramatic phosphorylation with a similar magnitude and time course with Akt. In the presence of GA, the TNF- $\alpha$ -induced increase in Akt phosphorylation was largely reduced, while increased GSK-3 $\beta$  phosphorylation was not affected (Figure 2A and 2B). To further determine whether the inhibition of the TNF- $\alpha$ -induced invasion by GA is mediated by Akt signaling pathway, we evaluated the effect of the PI3K/Akt pathway inhibitor on *in vitro* migration and invasion. We found that treatment with LY294002 strongly suppressed PC3 cell migration and invasion induced by TNF- $\alpha$ , which is identical to the effect of GA (Figure 3). This result suggested that the PI3K/Akt signaling pathway may be involved in the anti-invasion effect of GA in PC3 cells.

### Suppression of TNF- $\alpha$ -induced NF- $\kappa$ B transcriptional activity and NF- $\kappa$ B nuclear translocation by GA

We then addressed whether GA influences TNF- $\alpha$ -induced NF- $\kappa$ B transcriptional activity in PC3 cells using an NF- $\kappa$ B-mediated luciferase reporter gene assay. Our results demonstrated that TNF- $\alpha$  strongly induced luciferase activity of endogenous NF- $\kappa$ B compared to the basal activity in non-stimulated control cells. However, delivery of GA dose-dependently reduced TNF- $\alpha$ -mediated NF- $\kappa$ B transcriptional activity (Figure 4A). Under inflammatory stimulation, dissociation of I $\kappa$ B from the I $\kappa$ B/NF- $\kappa$ B complex leads to NF- $\kappa$ B p65 subunit translocation into the nucleus where it binds to specific promoter sites on target genes<sup>[17]</sup>. To investigate whether the anti-invasion effect of GA is mediated by inhibition of



**Figure 2.** Effect of Gambogic acid (GA) on TNF- $\alpha$ -mediated activation of PI3K/Akt/GSK-3 $\beta$  pathway. (A) PC3 cells were treated with TNF- $\alpha$  (10 ng/mL) for indicated time. The active phosphorylated forms of Akt and GSK-3 $\beta$  or Akt, GSK-3 $\beta$  and GAPDH were analyzed by Western blot analysis. (B) Cells were pretreated with GA (0.5  $\mu$ mol/L) or the specific PI3K inhibitor LY294002 (5  $\mu$ mol/L) for 2 h and then treated with TNF- $\alpha$  (10 ng/mL) for an additional 2 h. The active phosphorylated forms of Akt and GSK-3 $\beta$  were determined using Western blot analysis. Data are representative images or expressed as the mean $\pm$ SD of each group of cells from three independent experiments. <sup>b</sup> $P$ <0.05 vs control group; <sup>d</sup> $P$ >0.05, <sup>e</sup> $P$ <0.05 vs TNF- $\alpha$  alone treatment group.



**Figure 3.** Effect of LY294002 on TNF- $\alpha$ -induced migration and invasion. PC3 cells ( $5 \times 10^4$ ) were cultured in the upper compartment and incubated with GA (0.5  $\mu\text{mol/L}$ ) or LY294002 (5  $\mu\text{mol/L}$ ) for 12 h (migration assay) or 24 h (invasion assay). The TNF- $\alpha$ -induced migrating or invading cells were counted in a blind manner. Data are representative images or expressed as the mean  $\pm$  SD of each group of cells from three independent experiments. <sup>b</sup> $P < 0.05$  vs control group; <sup>e</sup> $P < 0.05$  vs TNF- $\alpha$  alone treatment group. Imaged at 200 $\times$  magnification.

TNF- $\alpha$ -induced NF- $\kappa$ B activation, we evaluated the nuclear translocation of NF- $\kappa$ B p65 using Western blot and immunofluorescence analyses. Western blot and immunofluorescence analyses revealed that TNF- $\alpha$  induced an obvious increase in NF- $\kappa$ B p65 nuclear translocation in comparison to the control group. However, this increased NF- $\kappa$ B nuclear translocation was significantly suppressed by the treatment with GA (Figure 4B and 4C).

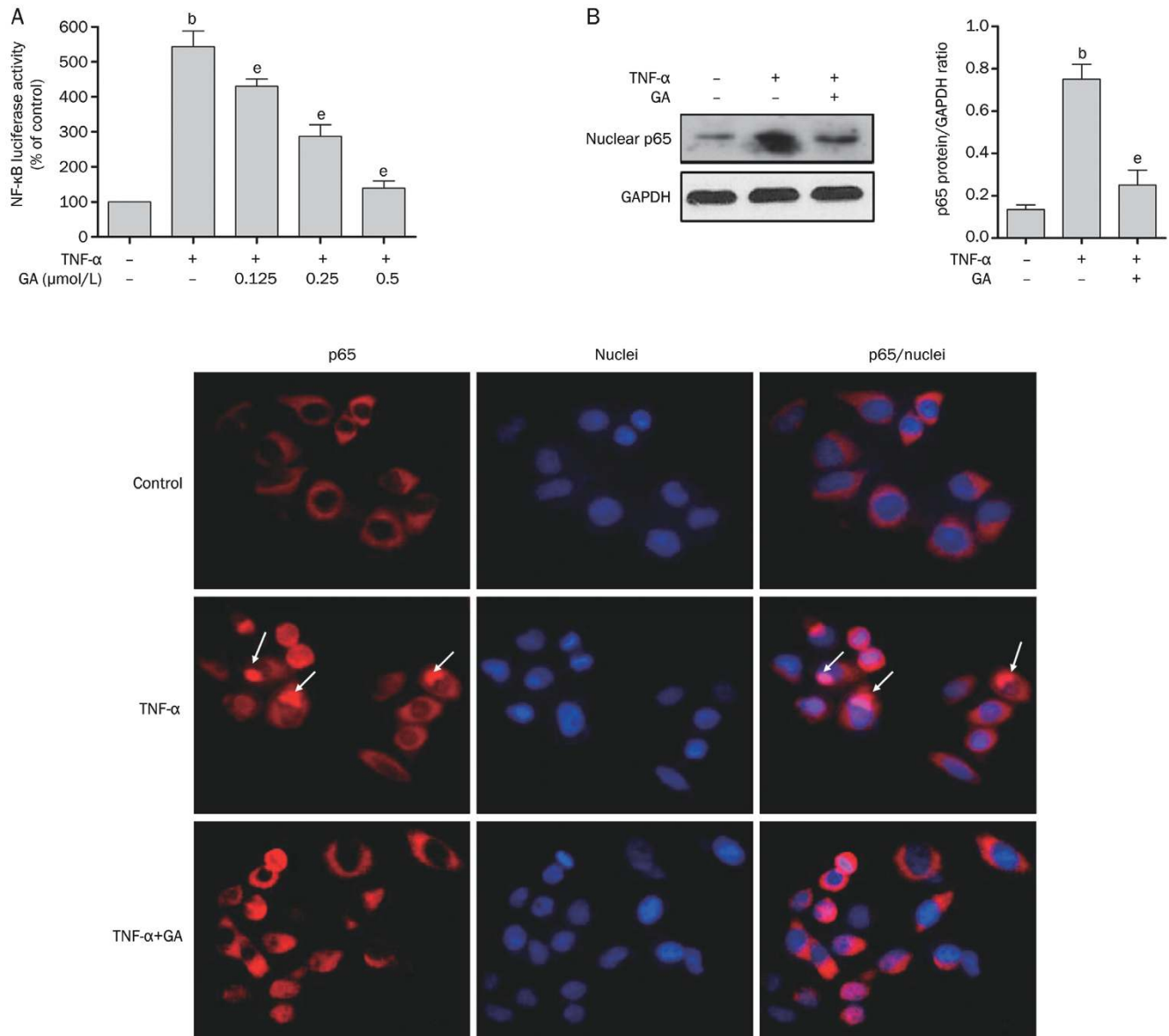
#### GA represses the TNF- $\alpha$ -induced physical interaction between the p65 subunit of NF- $\kappa$ B and Snail in PC3 cells

Snail, an important mesenchymal molecular marker, is known to increase tumor cell invasion and contributes to the induction of the epithelial to mesenchymal transition (EMT)<sup>[18]</sup>. To investigate whether TNF- $\alpha$ -stimulation could affect the expression of Snail, we determined the expression levels of Snail mRNA and protein using qRT-PCR and Western blot analysis, respectively. Upon TNF- $\alpha$ -stimulation, both Snail mRNA and protein displayed an up-regulated expression in PC3 cells compared to the control group. However, administration of GA was found to markedly reduce the increase of Snail mRNA and protein levels induced by TNF- $\alpha$  (Figure 5A and 5B). It was recently reported that transcription of Snail was regulated

by the NF- $\kappa$ B pathway<sup>[6]</sup>. As a critical subunit of NF- $\kappa$ B, p65 can bind the human Snail promoter and increase Snail transcription<sup>[19]</sup>. To further determine the mechanisms underlying the regulatory effect of GA on TNF- $\alpha$ -induced cell invasion, we examined the DNA binding activity of NF- $\kappa$ B using a ChIP assay. Our data revealed that TNF- $\alpha$ -stimulation significantly increased p65 binding to the Snail promoter. However, the TNF- $\alpha$ -mediated NF- $\kappa$ B DNA binding activity was inhibited by treatment with GA (Figure 5D).

#### Knockdown of Snail by RNAi inhibits TNF- $\alpha$ -induced cell invasion

Because the expression level of Snail was up-regulated by TNF- $\alpha$ -stimulation, we investigated the effect of silencing Snail on TNF- $\alpha$ -induced cell invasion. It has been previously shown that Snail overexpression could induce tumor progression via regulation of invasion, migration and EMT in a variety of cancer cell lines<sup>[20]</sup>, which can be attenuated by a knockdown of Snail. We therefore assessed the role of Snail on TNF- $\alpha$ -induced cell invasion with RNA interference. As expected, transfection with specific Snail siRNA-1 dramatically inhibited TNF- $\alpha$ -mediated cell invasion consistent with the decreased expression levels of Snail (Figure 6). When compared to the GA treatment group, the Snail siRNA-1



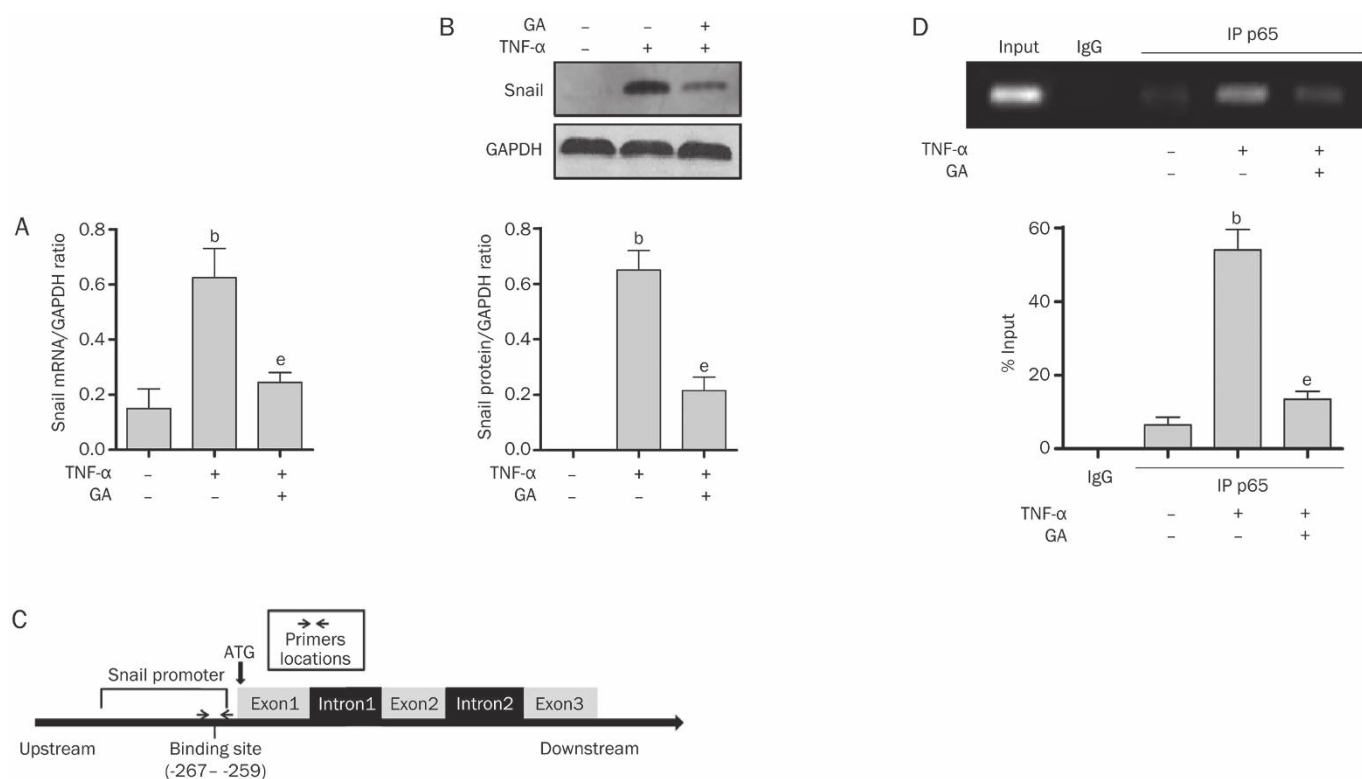
**Figure 4.** Effect of GA on TNF- $\alpha$ -induced activation of NF- $\kappa$ B. (A) PC3 cells were transiently co-transfected with pGL6-NF- $\kappa$ B-TA-luc and pRL-TK. After 6 h, the cells were incubated with TNF- $\alpha$  (10 ng/mL) for 12 h. Then, the cells were treated with or without GA for additional 12 h. The transcriptional activity of NF- $\kappa$ B was determined by luciferase reporter gene assay and normalized to the internal control. (B) PC3 cells were pretreated with TNF- $\alpha$  (10 ng/mL) for 12 h and incubated with or without GA (0.5  $\mu$ mol/L) for additional 12 h. Cells were then harvested and cellular nuclear protein extracted using Nuclear and Cytoplasmic Extraction Reagents kit according to the manuscript's instructions. Overall, 30  $\mu$ g of the nuclear proteins was resolved by SDS-PAGE on 10% polyacrylamide gels and transferred to nitrocellulose membranes. The blots were probed with anti-NF- $\kappa$ B p65 or anti-GAPDH antibodies. (C) The nuclear translocation was analyzed by immunofluorescence assay. Data are representative images or expressed as the mean $\pm$ SD of each group of cells from three independent experiments. <sup>b</sup> $P$ <0.05 vs control group; <sup>e</sup> $P$ <0.05 vs TNF- $\alpha$  alone treatment group. Imaged at 200 $\times$  magnification.

group exhibited a similar effect to GA in inhibiting the expression levels of Snail, cell invasion and migration stimulated by TNF- $\alpha$  (Figure 6C–6E). These data further supported that the regulation of Snail appears to be responsible for the inhibition of TNF- $\alpha$ -induced cell invasion by GA.

#### Modulation of TNF- $\alpha$ -induced invasiveness-associated genes expression by GA

Considering that Snail regulates the expression of inva-

siveness-associated genes, such as E-cadherin, MMP-9, and fibronectin<sup>[21, 22]</sup>, we determined whether GA could regulate the expression of invasiveness-associated genes in TNF- $\alpha$ -stimulated PC3 cells. It was demonstrated that TNF- $\alpha$ -treated PC3 cells exhibited up-regulated mRNA and protein expression levels of both MMP-9 and fibronectin. In contrast, the expression of E-cadherin was down-regulated by TNF- $\alpha$ . However, incubation of cells with GA or Snail siRNA effectively blocked the TNF- $\alpha$ -induced upregulation of MMP-9 and



**Figure 5.** Effect of GA on TNF- $\alpha$ -induced physical interaction between p65 and Snail. (A, B) Cells were treated with TNF- $\alpha$  (10 ng/mL) for 12 h, and the expression of Snail mRNA and protein was determined by qRT-PCR and western blot analysis, respectively. (C) Schematic diagram showing positions of putative Snail promoter and NF- $\kappa$ B p65 binding site. The upstream DNA sequence of the Snail gene and putative Snail promoter sequence were analyzed using the Promoter Retrieval online database (<http://rulai.cshl.edu/cgi-bin/TRED>). The binding site of the putative Snail promoter was obtained by gene regulation available online (<http://www.gene-regulation.com>). (D) Cells were pretreated with TNF- $\alpha$  (10 ng/mL) for 12 h and then incubated with or without GA (0.5  $\mu$ mol/L) for an additional 12 h. The ability of p65 to bind to the Snail promoter was detected using a ChIP assay. ChIP was performed using antibodies against p65 and IgG. NF- $\kappa$ B p65-immunoprecipitated DNA was amplified with PCR. Data are representative images or expressed as the mean $\pm$ SD of each group of cells from three independent experiments. <sup>b</sup> $P$ <0.05 vs control group; <sup>e</sup> $P$ <0.05 vs TNF- $\alpha$  alone treatment group.

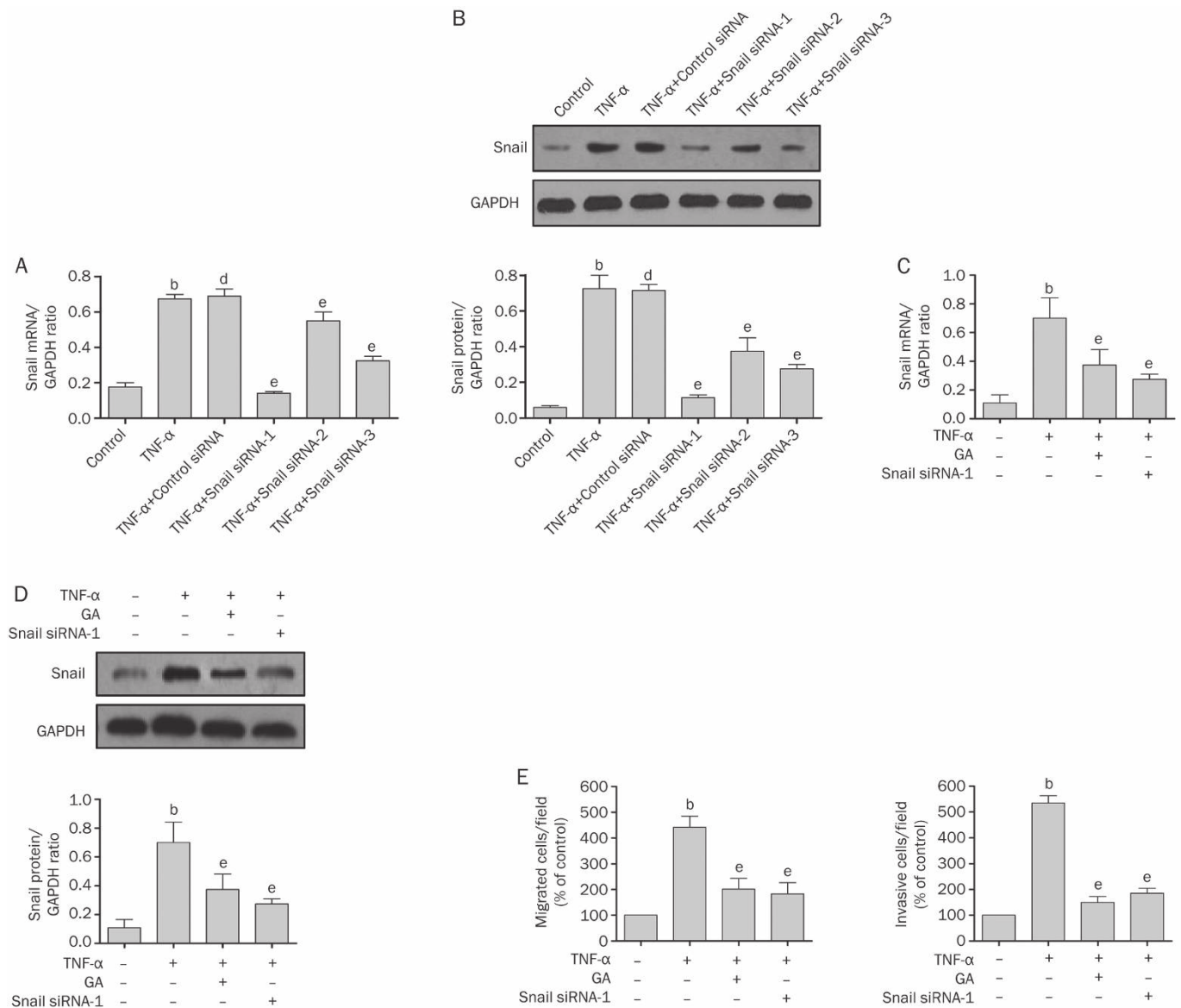
fibronectin and downregulation of E-cadherin (Figure 7A and 7B).

## Discussion

Prostate cancer is the most common urological neoplasm and the major cause of cancer-related death in aging men<sup>[23]</sup>. An important factor for the mortality rate of prostate cancer is the invasiveness of the constituent tumor cells causing metastasis<sup>[24]</sup>. Prostate cancer usually progresses through hormone-dependent to hormone-independent stages, leading to a low efficacy for anti-androgen therapies and increasing the probability of metastasis<sup>[25]</sup>. Despite numerous efforts that have focused on investigating the processes of tumor invasion and metastasis for the development of new therapies, the precise mechanisms causing the directional migration and invasion of tumor cells into selective organs is poorly understood. Thus, development of effective anti-metastatic therapies for prostate cancer will be of great significance.

Inflammation has been confirmed to play a crucial role in cancer progression including prostate cancer<sup>[26]</sup>. It is assumed that inflammation could promote survival or induce the death

of tumor cells through various signaling pathways<sup>[27]</sup>. In some cancers, inflammation was identified as a tumor promoting factor, which might increase the risk of tumor metastasis<sup>[28]</sup>. TNF- $\alpha$  has been demonstrated to be an important inflammatory cytokine produced by immune cells under inflammation. Although some studies reported its capacity to induce tumor necrosis<sup>[29, 30]</sup>, accumulating evidence has shown that TNF- $\alpha$  could induce tumor cell invasion and migration as an endogenous tumor promoter<sup>[31-34]</sup>. Overproduction of TNF- $\alpha$  disrupted cell adhesion and increased the metastatic potential of tumor cells. In addition, it is worthwhile to mention that TNF- $\alpha$  might increase the risk of cancer development and spreading in the specific tumor microenvironment<sup>[35]</sup>. GA, a major active ingredient of gamboges, has been recently demonstrated to possess potent anti-cancer activity in several tumor cell lines<sup>[9-13]</sup>. Previous reports have shown that GA could induce cell apoptosis and repress proliferation via several mechanisms, such as inhibiting the NF- $\kappa$ B signaling pathway, regulating the expression of Bcl-2 and Bax and disturbing CDK7-mediated phosphorylation of CDC2/p34<sup>[9, 10, 12]</sup>. As an NF- $\kappa$ B inhibitor, GA could enhance the pro-apoptotic



**Figure 6.** Effect of Snail silencing on the TNF- $\alpha$ -induced migration and invasion of PC3 cells. (A, B) PC3 cells were pretreated with TNF- $\alpha$  (10 ng/mL) for 12 h and then transfected with control siRNA or Snail siRNAs for 24 h. The mRNA and protein expression levels of Snail were determined by qRT-PCR and Western blot analysis, respectively. (C, D) PC3 cells were treated with the Snail-specific siRNA-1 for 24 h or GA for 12 h, and the TNF- $\alpha$ -induced mRNA and protein expression levels of Snail were measured by qRT-PCR and Western blot analysis, respectively. (E) Cells were treated with the Snail-specific siRNA-1 for 24 h or GA for 12 h, and TNF- $\alpha$ -induced migration and invasion were measured by migration and invasion assays. Data are representative or expressed as the mean $\pm$ SD of each group of cells from three independent experiments. <sup>b</sup> $P$ <0.05 vs control group; <sup>e</sup> $P$ >0.05 vs TNF- $\alpha$  alone treatment group; <sup>e</sup> $P$ <0.05 vs TNF- $\alpha$  alone treatment group.

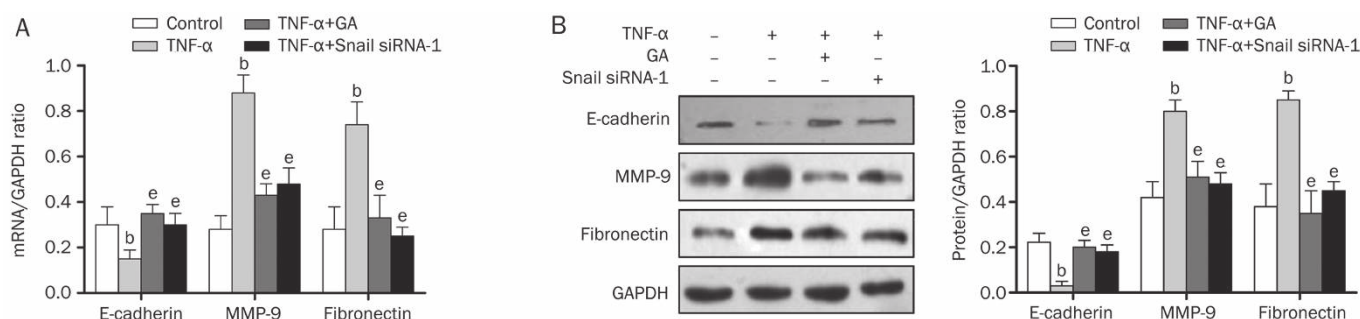
and anti-proliferative effects of TNF- $\alpha$ <sup>[36]</sup>. However, it is still unclear whether GA has anti-invasion properties under the chronic inflammatory condition induced by TNF- $\alpha$  in prostate cancer.

In the present study, we have demonstrated that TNF- $\alpha$ -induced migration and invasion of PC3 cells were significantly suppressed by GA. Moreover, TNF- $\alpha$ -induced cell invasion was associated with activation of the PI3K/Akt and NF- $\kappa$ B, and such activation was inhibited by treatment with GA. Furthermore, GA inhibited the TNF- $\alpha$ -induced cell invasion and

migration by regulating the expression of Snail.

Akt is a critical component of signal transduction following PI3K activation, which has been linked to cell survival and development<sup>[16, 37]</sup>. An accumulating body of evidence revealed that the PI3K/Akt signaling pathway was involved in morphological changes, migration and invasion induced by TNF- $\alpha$  in various cell types. Activated Akt-phosphorylated substrates can directly or indirectly affect downstream transcription factor activity, including NF- $\kappa$ B<sup>[38]</sup>. Thus, Akt activity may be essential for TNF- $\alpha$ -induced oncogenic transformation





**Figure 7.** GA regulates the expression of invasiveness-associated genes in TNF- $\alpha$ -stimulated PC3 cells. (A, B) Cells were incubated with TNF- $\alpha$  (10 ng/mL) for 12 h and then treated with GA for 12 h or the Snail-specific siRNA-1 for 24 h. The mRNA and protein expression levels of E-cadherin, MMP-9, and fibronectin were assessed by qRT-PCR and Western blot analysis, respectively. Data are representative images or expressed as the mean $\pm$ SD of each group of cells from three independent experiments. <sup>b</sup> $P$ <0.05 vs control group; <sup>e</sup> $P$ <0.05 vs TNF- $\alpha$  alone treatment group.

in cancer cells, and the treatment of prostate cancer cells with Akt inhibitors blocks the progression of tumorigenesis<sup>[37, 39, 40]</sup>. Our results further supported that TNF- $\alpha$ -induced invasion is mediated by Akt. More importantly, GA greatly reduced the phosphorylation and activation of Akt in PC3 cells. GSK-3 $\beta$  is the downstream target of the PI3K signaling pathway, which has also been shown to regulate cell survival, proliferation and differentiation<sup>[41]</sup>. In the present study, we found that TNF- $\alpha$  induced the phosphorylation of GSK-3 $\beta$ . However, GA does not affect TNF- $\alpha$ -activated GSK-3 $\beta$  in PC3 cells, indicating that GA-mediated inhibition of migration and invasion is independent of GSK-3 $\beta$  phosphorylation.

NF- $\kappa$ B plays a critical role in TNF- $\alpha$ -induced tumor development. It has been previously reported that the NF- $\kappa$ B signaling pathway is activated upon TNF- $\alpha$ -induced tumor cell migration and invasion via G protein coupled receptors. Several studies have shown that constitutive activation of NF- $\kappa$ B is associated with TNF- $\alpha$ -induced cell migration and invasion<sup>[6, 33, 40]</sup>. In the present study, utilizing western blot, immunofluorescence and luciferase reporter gene assays, we further confirmed that TNF- $\alpha$  can indeed increase NF- $\kappa$ B transcriptional activity. However, administration of GA can markedly inhibit TNF- $\alpha$ -induced NF- $\kappa$ B transcriptional activity in PC3 cells.

Several zinc-finger transcriptional repressors, including Snail, Slug, Twist, Zeb1, and Zeb2, have been shown to repress transcription of E-cadherin<sup>[18, 34, 42]</sup>. Snail was first identified as the most important transcriptional repressor of E-cadherin<sup>[43]</sup>. Snail can down-regulate expression of E-cadherin, inducing EMT and promoting cell migration and invasion<sup>[22, 44]</sup>. Over-expression of Snail was detected in various tumor cell lines, including breast, pancreatic and gastric cancers<sup>[45-47]</sup>. It was recently reported that Akt could up-regulate Snail expression and induce invasion<sup>[40, 48]</sup>. In addition, TNF- $\alpha$ -induced cell migration and invasion were associated with NF- $\kappa$ B-mediated stabilization of Snail<sup>[3, 6]</sup>. Because NF- $\kappa$ B may bind to the human Snail promoter causing increased Snail expression<sup>[19]</sup>, we tested whether the regulation of Snail expression might be involved in the TNF- $\alpha$ -mediated migration and invasion of PC3 cells. A CHIP assay revealed that the NF- $\kappa$ B p65 sub-

unit was constitutively bound to the Snail promoter, and this binding was increased upon NF- $\kappa$ B activation by TNF- $\alpha$ . The opposite effect was observed after GA addition. Given that Snail is the critical regulator of cell migration and invasion, we further investigated the impact of Snail knockdown on TNF- $\alpha$ -induced migration and invasion of PC3 cells. We surprisingly found that knockdown of Snail expression inhibited TNF- $\alpha$ -induced migration and invasion of PC3 cells. These data further supported that GA can down-regulate Snail expression, leading to an inhibition of TNF- $\alpha$ -induced tumor migration and invasion by PC3 cells. We subsequently examined the expression of Snail-regulated genes, such as E-cadherin, MMP-9 and fibronectin, which are associated with cell migration and invasion. As expected, both Snail siRNA and GA down-regulated MMP9 and fibronectin and upregulated E-cadherin expression in TNF- $\alpha$ -stimulated PC3 cells.

In summary, we showed that GA could inhibit TNF- $\alpha$ -induced migration and invasion. This inhibitory effect of GA is likely mediated by regulation of the PI3K/Akt and NF- $\kappa$ B signaling pathways resulting in a down-regulation of Snail. This finding may provide a new development of therapies for the inhibition TNF- $\alpha$ -induced migration and invasion of prostate cancer. Further studies *in vivo* are needed to determine the full potential of GA in prevention of cancer.

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### Author contribution

Lei LÜ, Liang WANG, and Fu-qing ZENG designed the research; Lei LÜ, Dong TANG, Guo-song JIANG, and Xing-yuan XIAO performed the research; Lei LÜ, Lu-qi HUANG, and Fu-qing ZENG analyzed the data and wrote the paper.

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