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# Gambogic acid inhibits tumor cell adhesion by suppressing integrin β1 and membrane lipid rafts-associated integrin signaling pathway

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ABSTRACT

Cell adhesion plays an important role in the steps of cancer metastasis. Regulation of cell–cell (intercellular) and cell–matrix adhesion is a promising strategy for cancer progression. Gambogic acid is a xanthone derived from the resin of the Chinese plant *Garciania hanburyi*, with potent anti-metastasis activity on highly metastatic cells. The aim of this study was to investigate the function and mechanism of gambogic acid on tumor adhesion. We found that gambogic acid strongly inhibited the adhesion of human cancer cells to fibronectin. This inhibition was associated with the deformation of focal adhesion complex, which was mediated by suppressing the expression of integrin β1 and integrin signaling pathway. *In vitro*, cell lipid rafts clustering was inhibited following treatment of gambogic acid, which induced the suppression of integrin β1 and focal adhesion complex proteins colocalization within rafts. Moreover, gambogic acid significantly decreased cellular cholesterol content, whereas cholesterol replenishment lessened the inhibitory effect of gambogic acid on cell adhesion. Real-time PCR analysis showed that gambogic acid reduced mRNA levels of hydroxymethylglutaryl-CoA reductase and sterol regulatory element binding protein-2, while increased acetyl-CoA acetyltransferase-1/2. Taken together, these results demonstrate that gambogic acid inhibits cell adhesion via suppressing integrin β1 abundance and cholesterol content as well as the membrane lipid raft-associated integrin function, which provide new evidence for the anti-cancer activity of gambogic acid.



## 1. Introduction

Cancer cells are characterized by uncontrolled growth, invasion into surrounding tissues, and in many cases metastatic spread to distant sites, which is the primary cause of cancer-related mortality. Tumor metastasis occurs by a complex series of events, including cell adhesion, invasion, proliferation, and vessel formation [1]. The invasion of tumor cells into adjacent tissues is a crucial event in metastasis. Invasion of tumor cells involves multiple processes, which depend on specific cell-to-cell and cell-to-extracellular matrix (ECM) interactions [2]. It is indicated that anti-adhesion is an effective strategy for metastasis inhibition [3,4]. Therefore, anti-adhesion as one of the targets for treating tumor metastasis has been widely accepted and extensively investigated [5].

Cell adhesion is mediated directly by specific adhesion receptors, such as integrins, cadherins, selectins, and intercellular adhesion molecules [6]. Integrins and their downstream signaling play an important role in cell adhesion. Integrin is an α/β heterodimeric membrane protein that mediates cell adhesion to

*Abbreviations:* ACAT, acetyl-CoA acetyltransferase; BSA, bovine serum albumin; CTxB, cholera toxin B subunit; ECM, extracellular matrix; FAC, focal adhesion complex; FAK, focal adhesion kinase; FITC, fluorescein-5-isothiocyanate; FN, fibronectin; GA, gambogic acid; GC–MS, gas chromatography–mass spectrometry; HMG, hydroxy-methylglutaryl; LDL-r, low density lipoprotein receptor; MMP, matrix metalloproteinase; MBCD, methyl-β-cyclodextrin; PE, phycoerythrin; SREBP, sterol regulatory element binding protein; SR-BI, scavenger receptor class B type I.

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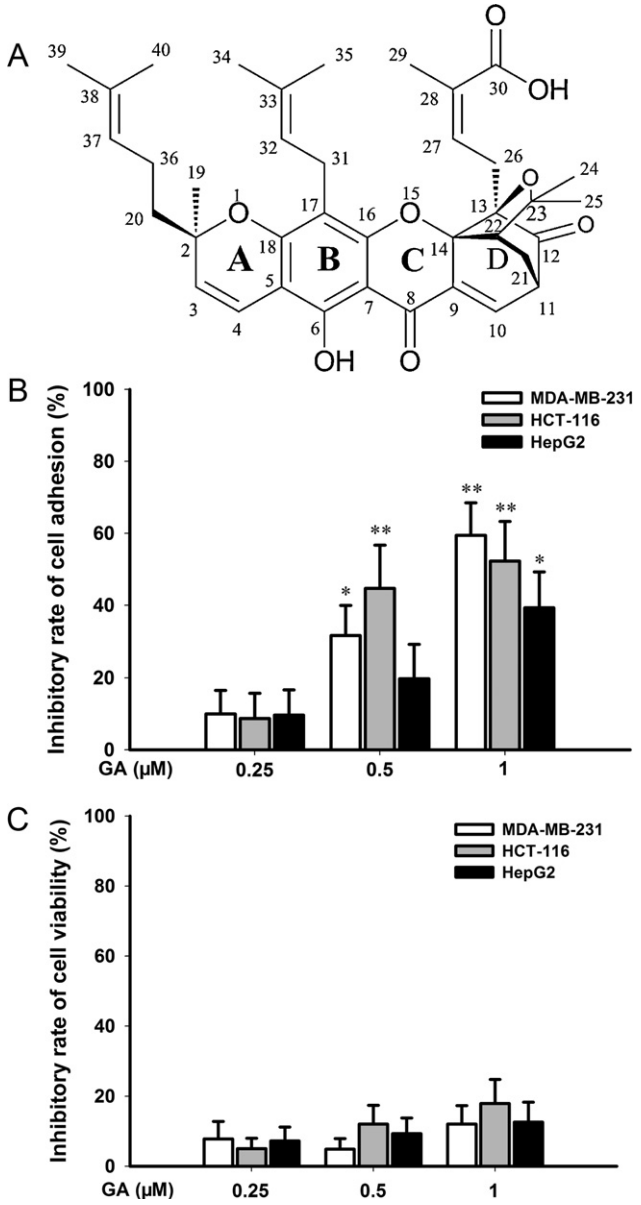


**Fig. 1.** GA inhibits cancer cell adhesion. (A) Chemical structure of GA. (B) Inhibitory effect of GA on adhesion of MDA-MB-231 cells, HCT-116 cells, and HepG2 cells to fibronectin. (C) Effect of GA on the viability of MDA-MB-231 cells, HCT-116 cells, and HepG2 cells. $^{*}P < 0.05$ or $^{**}P < 0.01$ represents significant difference from the control group.

components of the ECM. Integrin β1 subunit is crucial for adhesion to fibronectin (FN) [7], one important component of ECM. With interaction of ECM components, integrin molecules cluster at specific locations in the plasma membrane [8], which mediates transmembrane signal transduction via other signaling molecules recruited to focal adhesions [9], such as focal adhesion kinase (FAK), the kinase c-Src, paxillin, and vinculin [10]. It is also reported that integrin β1 is distributed in lipid rafts [11]. Lipid rafts (also known as detergent-resistant membrane or detergent-insoluble membrane) are at the cell surface membrane by being clustered with sphingomyelin and/or cholesterol [12]. Lipid rafts have been suggested to be important for modulation of signal transduction and cell adhesion [13]. The function of integrins, such as integrin α5β1, is positively regulated by lipid rafts when associated with fibronectin [14]. This regulatory effect of lipid rafts is also found on integrin α4β1 [15].

Gambogic acid (GA, Fig. 1A) is the major active ingredient of gamboges, a brownish to orange resin from the Chinese plant, *Garcinia hanburyi*. Previous studies demonstrated the molecular mechanism of apoptosis activation effect of GA by binding to the transferrin receptor [16] and suppressing nuclear factor-kappa B signaling pathway [17], as well as the anti-angiogenesis effect by suppressing vascular endothelial growth factor receptor 2 signaling [18]. Our work indicated the potent anti-metastasis activity of GA, which mainly attributed to its inhibition of matrix metalloproteinase (MMP)-2 and MMP-9 [19,20]. GA also inhibits integrin α4-mediated adhesion of B16-F10 cells [21]. Since cell adhesion is a key step in the metastatic cascade of cancers, it is essential to investigate the effect of GA on cancer cell adhesion and the mechanism involved in. These findings provide new evidence of the anti-metastatic activity of GA.

## 2. Materials and methods

### 2.1. Materials

GA (99% purity) was supplied by Dr. Qidong You's laboratory (China Pharmaceutical University, China), which was isolated from the resin of *Garciania hanburyi* and purified according to the established methods [22]. GA was prepared as described previously [19]. Fibronectin was from BD Biosciences (Bedford, MA, USA). Antibodies to integrin β1 (8A2) (sc-73645), FAK (H-1) (sc-1688), p-FAK (Tyr397)-R (sc-11765-R), paxillin (H-114) (sc-5574), vinculin (H-300) (sc-5573), c-Src (N-16) (sc-19), p-c-Src (9A6) (sc-81521), β-actin (9) (sc-130301), isotype-matched immunoglobulin G (IgG) (ICO-97) (sc-66186), and Protein A/G Plus-Agarose were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The cholesterol standard preparation (purity >99%), methyl-β-cyclodextrin (MBCD), bovine serum albumin (BSA), MTT, paraformaldehyde, Triton X-100, Tris, NaCl, EDTA, NP-40, PMSF, NaF, SDS, DTT, and fluorescein-5-isothiocyanate (FITC)-conjugated cholera toxin B subunit (CTxB) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

### 2.2. Cell culture

MDA-MB-231 (a human breast carcinoma cell line), HCT-116 (a human colon carcinoma cell line), and HepG2 (a human hepatocarcinoma cell line) were purchased from the Cell Bank of the Shanghai Institute of Cell Biology (Shanghai, China). MDA-MB-231 cells, HCT-116 cells, and HepG2 cells were cultured in Leibovitz's L15, McCoy's 5A, and RPMI-1640 medium (Gibco, Invitrogen, Carlsbad, CA, USA), respectively, containing 10% fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin (Beyotime, Nantong, China), and 100 μg/mL streptomycin (Beyotime, Nantong, China). The cells were maintained in a humidified atmosphere of 95% air/5% $CO_2$ at 37 °C.

### 2.3. Cell adhesion assay

Cell adhesion assay was performed as described [21] with modifications. Ninety-six-well plates (BD Biosciences, Bedford, MA, USA) were coated with fibronectin (5 μg/mL) at 4 °C for overnight and then blocked in BSA (1%) for 1 h. Serum-starved cells were exposed to GA (0.25, 0.5, or 1 μM) for 24 h before seeding. Target cells were harvested and suspended in fetal bovine serum-free medium. Cells ($2 \times 10^5$ $mL^{-1}$) were seeded to fibronectin-coated plates and then incubated for 1 h at 37 °C. Non-adherent cells were removed by gentle washing with PBS. Then, colorimetric MTT assay was employed to analyze the adhesion ability of cells.

### 2.4. Cell viability assay

Cells were seeded onto Falcon 96-well plates (BD Biosciences, Bedford, MA, USA) for 24 h. Then cells were exposed to GA (0.25,

0.5, or 1 μM) for 24 h. The colorimetric MTT assay was employed to determine the cell viability.

### 2.5. Immunofluorescence

Cells were pretreated with GA (1 μM) for 24 h, harvested, and then seeded onto fibronectin-coated or uncoated coverslips [23]. Following steps were performed as described [20]. Cells were fixed with 4% paraformaldehyde in PBS at 1-h intervals, permeabilized with 0.5% Triton X-100, and blocked with 2% BSA for 30 min. Incubation with primary antibodies (diluted 1:50) against FAK, paxillin, and vinculin was done overnight at 4 °C. After washing, cells were exposed to FITC-conjugated secondary antibodies (1:1000, Invitrogen, Carlsbad, CA, USA, M30101, L42001). Images were observed and captured with a fluorescence microscope (IX51, Olympus Corp., Tokyo, Japan).

### 2.6. Flow cytometry

Cells were pretreated with GA (0.25, 0.5, or 1 μM) for 24 h and seeded onto fibronectin-coated or uncoated Petri dishes (Corning, New York, NY, USA) for 1 h. Equivalent numbers of cells were fixed with 4% paraformaldehyde in PBS and then incubated with anti-integrin β1 (1 μg/1 × $10^6$ cells) for 1 h. As a negative control, cells were stained with IgG. Cells were then incubated with FITC-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA, M30101) for another 1 h and suspended in 500 μL PBS for flow cytometry analysis [24]. Relative amounts of cell surface integrins were determined with a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) and Cell Quest software (BD Biosciences, San Jose, CA, USA).

### 2.7. Western blotting analysis

Cells were pretreated with GA (0.25, 0.5, or 1 μM) for 24 h, seeded onto fibronectin-coated or uncoated Petri dishes for 1 h, and collected. The cells were lysed and Western blotting analysis for proteins was conducted according to previous methods [19]. Detection was performed with the Odyssey Infrared Imaging System (LI-COR Inc., Lincoln, NE, USA). All blots were stripped and reprobed with polyclonal anti-β-actin to verify equal protein loading.

### 2.8. Coimmunoprecipitation

Cells were pretreated with GA (0.25, 0.5, or 1 μM) for 24 h and seeded onto fibronectin-coated or uncoated Petri dishes for 1 h and collected. Then cells were lysed with coimmunoprecipitation lysis buffer (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% (w/v) NP-40, 0.2 mM PMSF, 0.1 mM NaF). Cell lysates (500 μg) were first incubated with 5 μg designated antibodies for 1 h at 4 °C followed by incubation with Protein A/G Plus-Agarose for another 1 h at 4 °C [25]. The beads were washed three times with ice-cold coimmunoprecipitation lysis buffer, resolved by SDS-PAGE, and immunoblotted with antibodies against various proteins of interest.

### 2.9. Immunocytochemical staining and visualization of lipid rafts

The labeling of membrane lipid rafts with CTxB was conducted as described [26] with slight modifications. Briefly, cells were pretreated with 1 μM GA for 24 h or 2 mM MBCD for 1 h and then seeded on fibronectin-coated or uncoated coverslips. After 1 h incubation, cells were fixed with 4% paraformaldehyde for 20 min followed by staining with FITC-conjugated CTxB (5 μg/mL) for 20 min. The double staining of integrin β1 was then carried out with phycoerythrin (PE)-conjugated goat anti-mouse IgG (1:1000, Invitrogen, Carlsbad, CA, USA, M30004-1). Cells were photographed with a Laser Scanning Microscope (LSM510 META, Carl Zeiss Inc., Germany).

### 2.10. Separation of proteins of detergent-insoluble fractions

Separation was conducted as described [27] with modifications. In brief, cells were pretreated with GA (0.25, 0.5, or 1 μM) for 24 h and seeded on fibronectin-coated or uncoated Petri dishes for 1 h. Cells were lysed in ice-cold lysis buffer (150 mM NaCl, 0.5% Triton X-100, 20 mM Tris, pH 7.5) containing proteinase inhibitor cocktail (Roche, Mannheim, Germany). The lysates were centrifuged at 14,000 × *g* for 30 min at 4 °C. Equivalent amounts of detergent-insoluble pellets were briefly sonicated in the same lysis buffer supplemented with 0.5% SDS and 2 mM DTT.

### 2.11. Analysis of cholesterol

Cells were treated with 1 μM GA for 24 h or 2 mM MBCD for 1 h followed by 30 min of cholesterol replenishment (30 μg/mL), and then plated on fibronectin-coated or uncoated Petri dishes for 1 h. Lipid extraction was done using the Bligh and Dyer method [28]. Cholesterol content was analyzed with gas chromatography–mass spectrometry (GC–MS, GCMS-QP2010, Shimadzu Corp., Kyoto, Japan) as described [29].

### 2.12. Real-time PCR analysis

Cells were treated with 1 μM GA for 24 h and then plated on fibronectin-coated Petri dishes for 1 h. The mRNA levels of hydroxymethylglutaryl (HMG)-CoA reductase, sterol regulatory element binding protein-2 (SREBP-2), acetyl-CoA acetyltransferase-1 (ACAT-1), and ACAT-2 were then determined. Primer sequences were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and shown in Supplementary Material. Total RNA was extracted with Tripure isolation reagent (Roche, Mannheim, Germany). The RT-PCR kit was purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Reactions were conducted with 2 μL of RT-PCR cDNA, 0.8 μL each forward and reverse primers (10 μM), 6 μL distilled/deionized dd$H_2O$, and 10 μL SYBR *Premix Ex Taq*™ (2×, TaKaRa, Dalian, China) with 0.4 μL Rox Reference DyeII (50×, TaKaRa, Dalian, China). Samples were run on the ABI 7500 Real-Time PCR system (ABI, Foster City, CA, USA) as follows: 30 s at 95 °C, followed by 40 cycles of 95 °C for 5 s, and 60 °C for 34 s. Each reaction was done in triplicate, and the threshold values (Cs) for each mRNA were subtracted from that of β-actin mRNA and averaged and converted from log-linear to linear terms. Data were analyzed with the SDS 2.1 program.

### 2.13. Statistical analysis

All data in the different experimental groups are expressed as the mean ± S.E.M. The data shown were obtained from at least three independent experiments. Differences between the groups were assessed by one-way ANOVA and Dunnett's post hoc test. Significant differences were represented as $^{*}P < 0.05$ or $^{**}P < 0.01$.

## 3. Results

### 3.1. GA inhibits the adhesion of cancer cells

Tumor cell adhesion to the ECM and basement membrane is one of the critical steps in metastasis. We examined the influence of GA on cancer cell adhesion to fibronectin. As shown in Fig. 1B, GA (1 μM) suppressed MDA-MB-231, HCT-116, and HepG2 cells adhesion to fibronectin by 60 ± 9% ($n = 3$, $P < 0.01$), 52 ± 11% ($n = 3$,

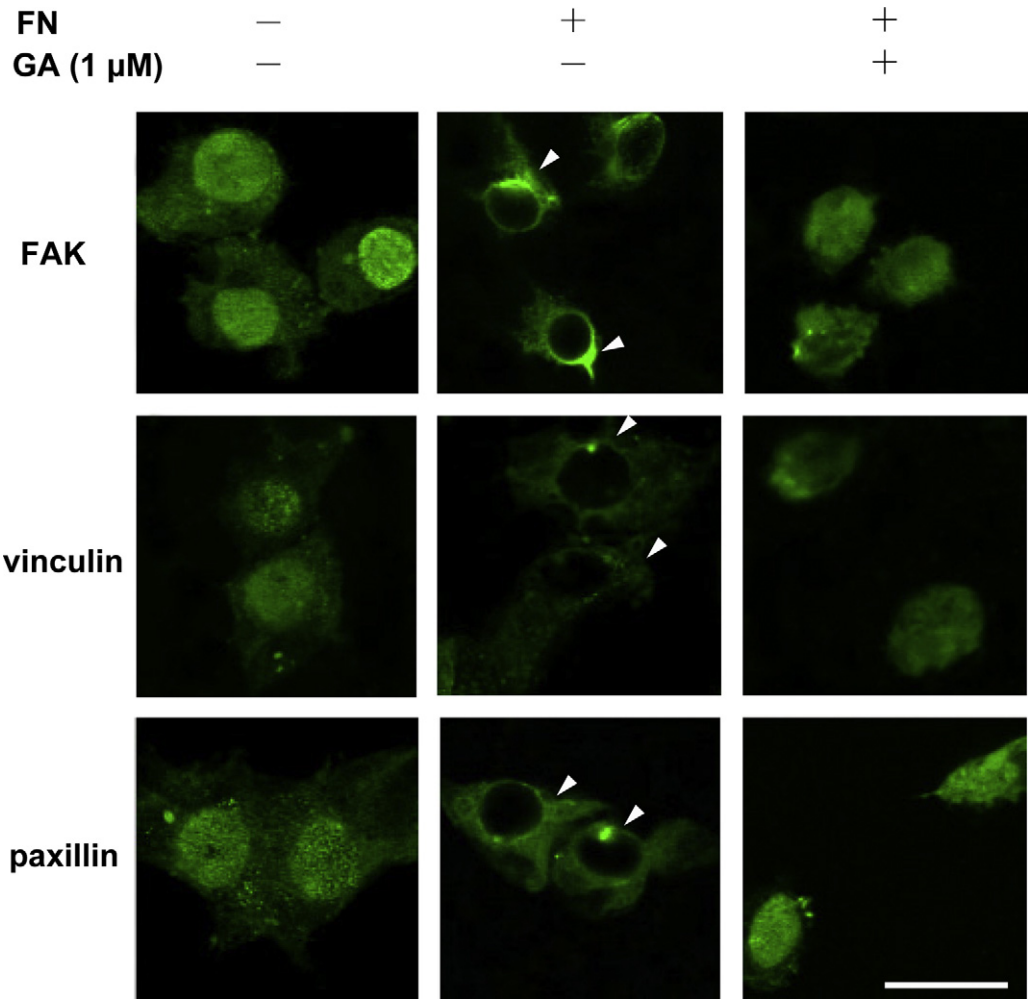


**Fig. 2.** GA alters focal adhesion formation. MDA-MB-231 cells were immunostained with anti-FAK, anti-vinculin, or anti-paxillin antibodies, 400×. (The scale bar is present as 30 μm.) Arrows indicate the clustering of focal adhesions.

$P < 0.01$), and $39 \pm 10\%$ ($n = 3$, $P < 0.05$), respectively. Importantly, GA (up to 1 μM) did not influence cell viability (Fig. 1C). Since MDA-MB-231 cell line is a high invasive cell line, which owns more significance for drug targeting, thus it was chosen for further mechanism studies.

### 3.2. GA alters the focal adhesion formation

Focal adhesion formation is an essential step during cell adhesion [10]. To investigate the underlying mechanism of the inhibitory effect on cell adhesion induced by GA, immunofluorescence staining was employed to examine the effect of GA on the distribution of FAK, vinculin, and paxillin, three major components of focal adhesions. According to previous results, we chose a relative higher concentration (1 μM) for investigation so as to demonstrate the effect of GA and the mechanism involved in more sufficiently. As shown in Fig. 2, FAK, vinculin, and paxillin were initially dispersed at both central and peripheral regions of cells. Following attachment to fibronectin for 1 h, however, focal adhesions were mainly at peripheral regions (arrows). Treatment with 1 μM GA altered the focal adhesion formation with adhesion proteins distribution mainly in central regions of cells.

### 3.3. GA decreases the expression of integrin β1

It is generally accepted that integrins mediate focal adhesion formation and subsequent cell attachment to ECM [30]. Therefore, the inhibitory effect of GA on adhesion may be achieved via the downregulation of integrin abundance at the cell surface and/or disruption of the integrin-mediated signaling pathway. Integrin β1 is crucial in adhesion to fibronectin and also cell migration [7]. Flow cytometry analysis showed that GA decreased integrin β1 expression on cell surface (Fig. 3A), and attachment to fibronectin had no significant effect in this regard. As shown in Fig. 3B, integrin β1 fluorescence intensity were inhibited by $7 \pm 4\%$ ($n = 3$, $P$ = NS), $21 \pm 7\%$ ($n = 3$, $P$ = NS), and $51 \pm 6\%$ ($n = 3$, $P < 0.01$), respectively, when the cells were treated with 0.25, 0.5, and 1 μM GA. These results were confirmed by Western blotting analysis (Fig. 3C).

### 3.4. GA blocks integrin signaling

Integrin signaling is the primary regulator of cell adhesion. It is well established that the recruitment of FAK to activated integrins is an early consequence of integrin-ligand interaction and is followed by rapid autophosphorylation of FAK at Tyr397 [31]. Thus, we used immunoprecipitation to examine the association between FAK and integrin β1. Fibronectin attachment significantly increased the association between FAK and integrin β1, which was expected because fibronectin may stimulate cells to activate adhesion-related signaling [23]. As shown in Fig. 4A, GA (0.5 μM and 1 μM) inhibited FAK-integrin β1 association, showing that the recruitment of FAK to integrin β1 was suppressed by GA. c-Src targets phosphorylated FAK and then fully phosphorylates and activates FAK [32]. Fibronectin enhanced the levels of p-FAK-

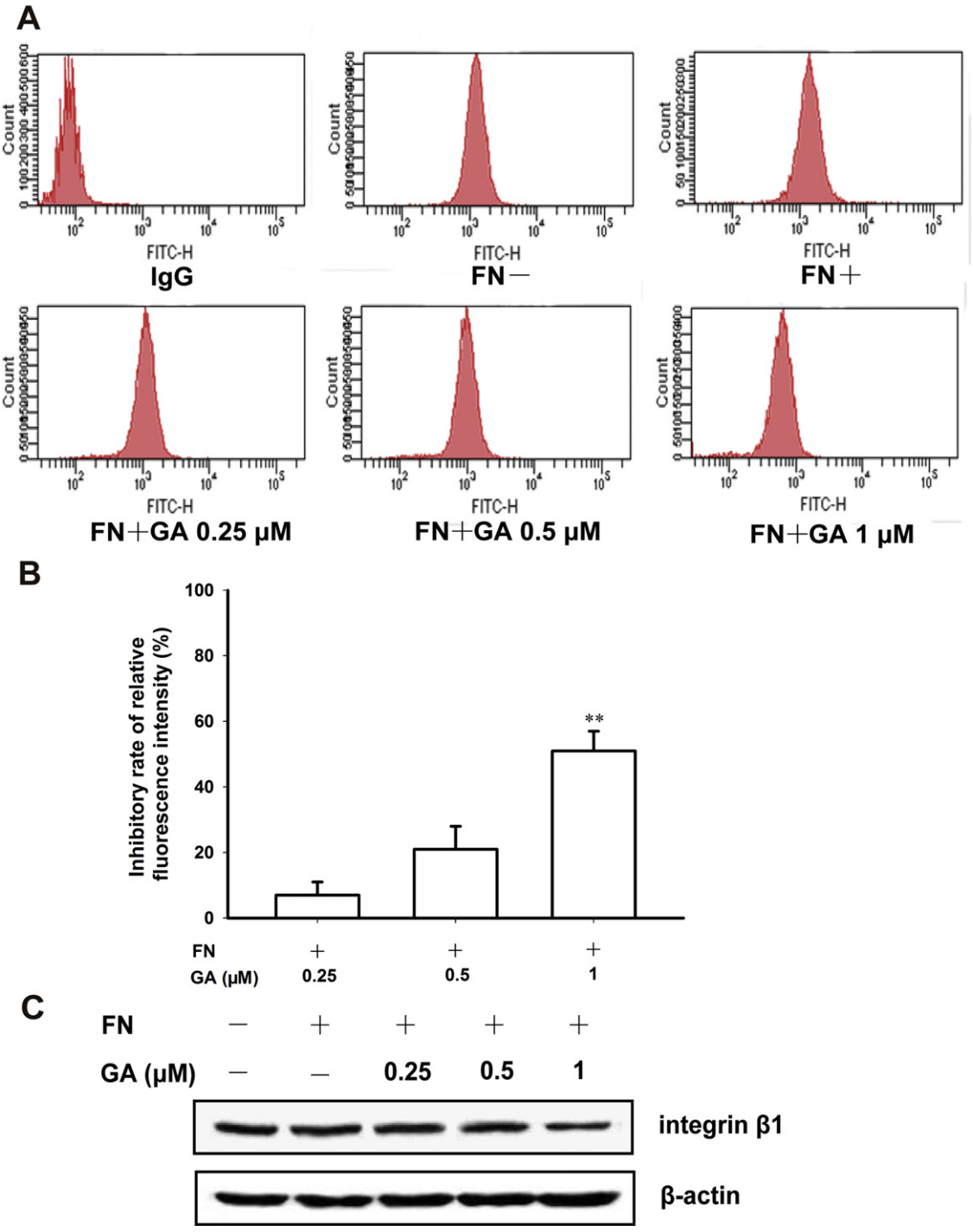


**Fig. 3.** GA decreases expression of integrin β1. (A) Flow cytometry analysis of integrin β1 abundance at the surface of MDA-MB-231 cells. (B) Quantification of the inhibitory rate of integrin β1 fluorescence intensity. $^{**}P < 0.01$ represents significant difference from the fibronectin group. (C) Western blotting analysis of the effect of GA on the expression of integrin β1 in MDA-MB-231 cells.

Tyr397 and p-c-Src although the total steady-state levels of FAK and c-Src remained unchanged (Fig. 4B). Treatment with GA (0.5 μM and 1 μM) efficiently inhibited the fibronectin-activated phosphorylation of FAK-Tyr397 and c-Src. GA (1 μM) decreased the total steady-state level of FAK by $30 \pm 8\%$ ($n = 3$, $P < 0.05$, Fig. 4C), whereas that of c-Src remained unchanged. These results indicated that GA-induced cell adhesion suppression also related to the regulation of integrin signaling.

Phosphorylated/activated FAK recruits additional structural and signaling molecules to assemble focal adhesion complexes (FACs), which are essential for cell adhesion. Therefore, we further examined whether GA inhibited direct protein–protein interactions within FACs. Paxillin and vinculin are two key molecules of focal adhesions [33]. As shown in Fig. 4D, GA had no effect on the levels of the paxillin and vinculin. The expressions were also remained unchanged in the presence of fibronectin, however, the association of FAK with paxillin and vinculin increased when cells were exposed to fibronectin. GA (0.5 μM and 1 μM) significantly inhibited the association of FAK with paxillin and vinculin. These data showed that GA disrupted the integrin β1-mediated signaling

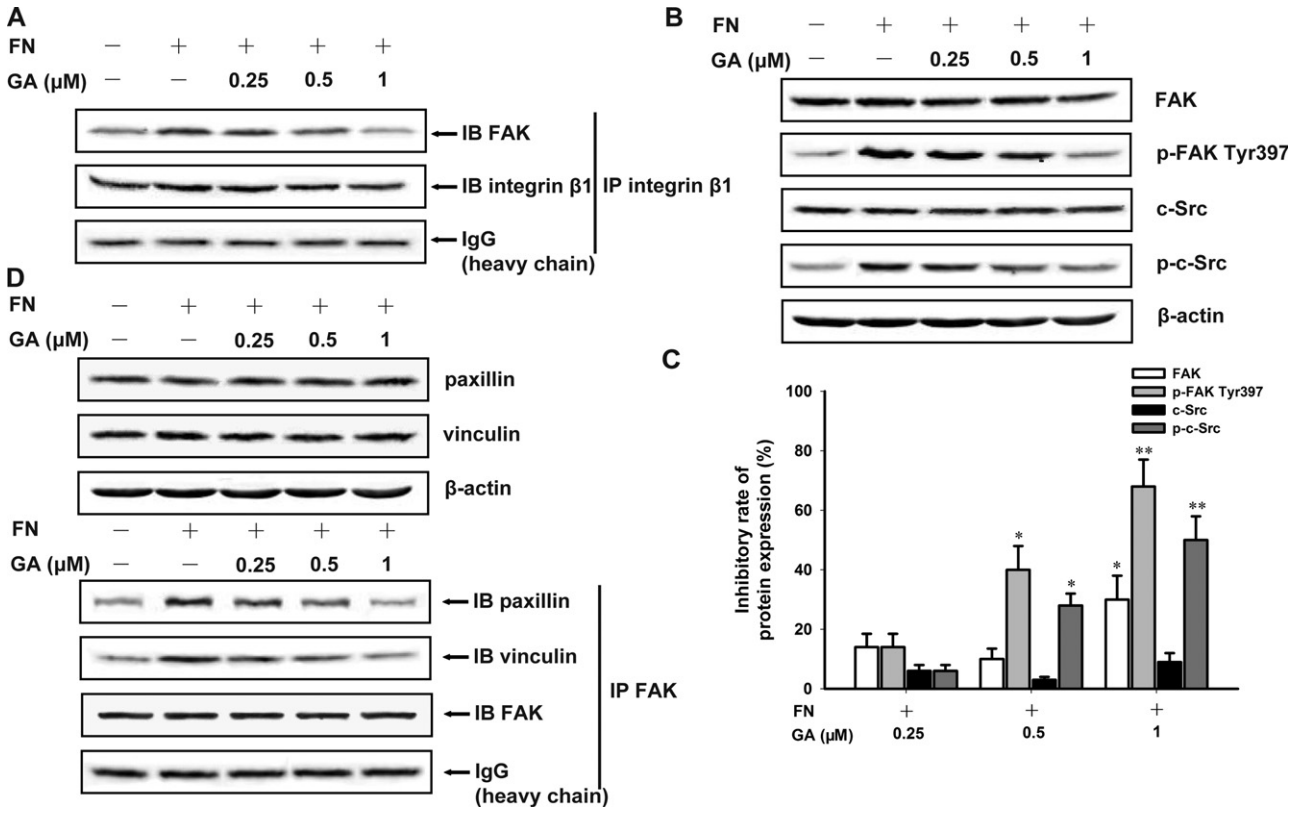


**Fig. 4.** GA suppresses recruitment of FAK to integrin β1, adhesion signaling, and formation of FACs. (A) GA suppresses recruitment of FAK to integrin β1. MDA-MB-231 cell lysates were immunoprecipitated (IP) with an antibody against integrin β1 and immunoblotted (IB) with an antibody against FAK (1:500). (B) GA inhibits signaling that normally promotes cell adhesion. MDA-MB-231 cell lysates were subjected to immunoblotting with antibodies against FAK (1:500), p-FAK Tyr397 (1:400), c-Src (1:500), and p-c-Src (1:400). (C) Densitometric analysis of the proteins studied (FAK, p-FAK Tyr397, c-Src, and p-c-Src). $^{*}P < 0.05$ or $^{**}P < 0.01$ represents significant difference from the fibronectin group. (D) GA suppresses FAC formation. Western blotting was used to analyze the expression of paxillin and vinculin. Cell lysates were then immunoprecipitated with an antibody against FAK and immunoblotted with an antibody against paxillin (1:500) or vinculin (1:500).

cascade by inhibiting the recruitment of FAK to integrin β1, then FAK activation, and subsequent FAC formation.

### 3.5. GA inhibits lipid raft clustering and association of integrin β1 with FAC proteins within lipid rafts

It has been reported that FAK recruitment to integrins is a consequence of integrin clustering [31]. Lipid rafts have been increasingly recognized as a key player in modulating integrin clustering via raft coalescence [34,35]. Therefore, we further examined the clusters of lipid rafts and integrin β1 and whether they colocalized at points of cell–matrix interactions. CTxB was used to visualize membrane rafts [26] because of its highly specific binding to raft ganglioside GM1. As shown in Fig. 5A, cell attachment to fibronectin led to significant coalescence of lipid rafts into distinctly larger patches with concurrent clustering of integrin β1 and colocalization of integrin β1 with lipid rafts (as arrows shown). GA (1 μM) markedly decreased the coalescence of lipid rafts, integrin β1 expression, as well as the clustering of integrin β1 with rafts patches. Interestingly, the inhibitory effect of GA on lipid raft clustering is similar to that of MBCD, an inhibitor of lipid raft formation [36]. These results indicated that GA also inhibited lipid raft and integrin β1 clustering, and then blocked FAC formation.

Next, we examined whether GA could affect the association of lipid rafts with integrins or with FAC molecules. Because lipid rafts are enriched with cholesterol and sphingolipids, they are insoluble after treatment with nonionic detergents such as Triton X-100 at 4 °C [27]. Thus, we examined the effects of GA on integrin β1, FAK, vinculin, and paxillin expression in detergent-insoluble fractions of cells. As shown in Fig. 5B, fibronectin attachment increased the expression of the focal adhesion molecules. Treatment with 1 μM GA reversed the effect induced by fibronectin. Besides, it is reported that c-Src is associated exclusively with membrane rafts [37], and GA had no effect on c-Src, which further confirmed the quality of the sample preparation in this study.

### 3.6. Cholesterol level involved in GA-mediated inhibition of cell adhesion

To further explore the disruptive effect of GA on lipid rafts, we employed GC–MS analysis to examine cholesterol content of cells, since cholesterol is one of the major components of lipid rafts [34]. The chromatogram results (selected ion monitoring $m/z$ 386) are shown in Fig. 6A. The cholesterol content was decreased by $81 \pm 5\%$ ($n = 3$, $P < 0.01$) following 1 μM GA treatment, which was similar to the effect of 2 mM MBCD ($91 \pm 2\%$, $n = 3$, $P < 0.01$; Fig. 6B). These results showed that GA reduced cellular cholesterol, leading to disruption of lipid rafts. We next performed cholesterol replenishment experiment to confirm the effect of GA on cholesterol dynamic balance. As shown in Fig. 6B, the cholesterol content was only decreased by $42 \pm 7\%$ or $13 \pm 3\%$ when cholesterol (30 μg/mL) was added to GA- or MBCD-treated cells, respectively. Moreover, the effects of GA on translocation of integrin β1 and FAC molecules to lipid rafts were also partially reversed by cholesterol replenishment (Fig. 7A). These results suggested that GA inhibit cell adhesion not only via downregulating integrin β1, but also through disrupting lipid raft-associated integrin signaling pathway by reducing cholesterol. This hypothesis was confirmed by the observation that cholesterol

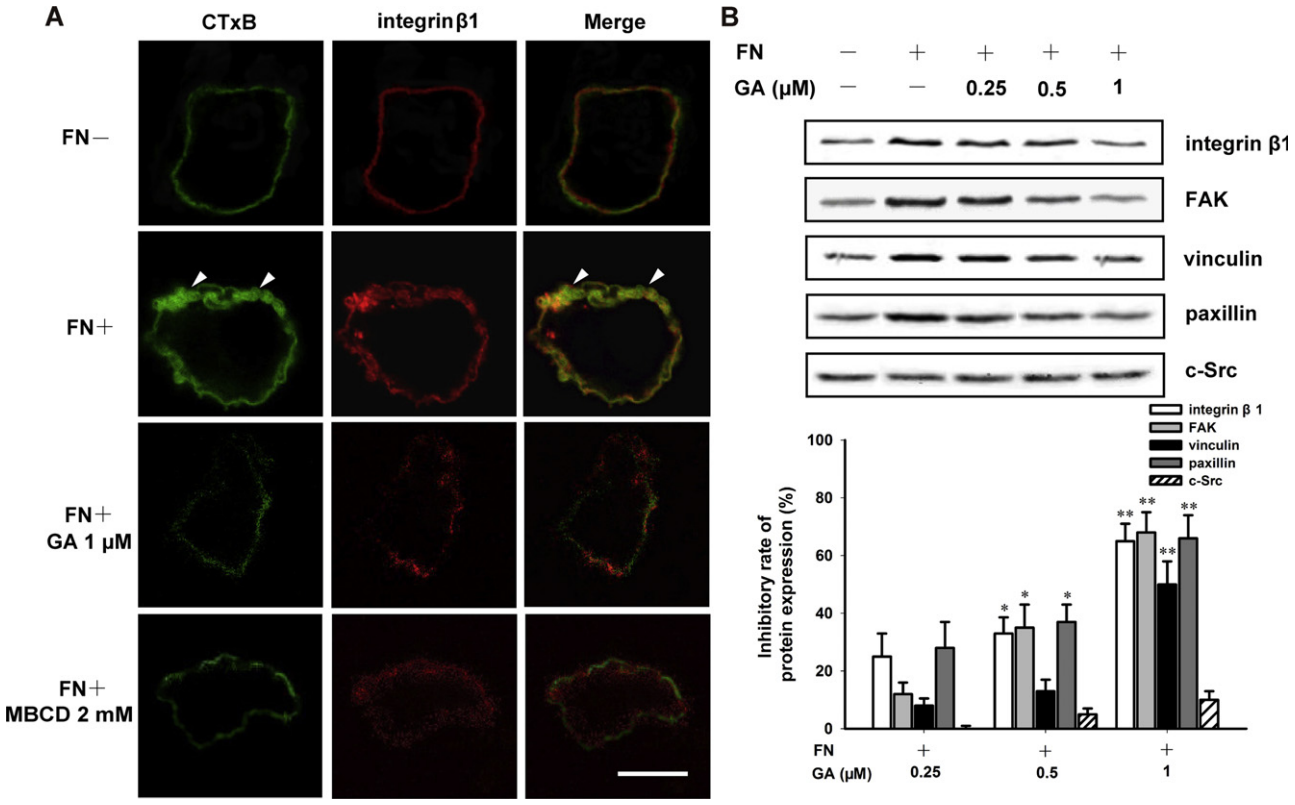


**Fig. 5.** GA inhibits clustering of lipid rafts and integrin β1, the colocalization of integrin β1 with lipid rafts, and the translocation of integrin β1 and FAC molecules to membrane lipid rafts. (A) GA inhibits lipid rafts coalescence and integrin β1 clustering. Representative cells are shown for each treatment, 600×. (The scale bar is present as 10 μm.) Arrows indicate the coalescence of lipid rafts or with integrin β1. (B) GA inhibits association of lipid rafts with integrin β1 or FAC molecules. Western blotting analysis was employed. $^{*}P < 0.05$ or $^{**}P < 0.01$ represents significant difference from the fibronectin group.

replenishment partially reversed the inhibitory effect on cell adhesion induced by 1 μM GA (Fig. 7B). Cell adhesion was only inhibited by 27 ± 9% or 20 ± 4% when cholesterol (30 μg/mL) was added to GA- or MBCD-treated cells, respectively.

### 3.7. GA regulates cholesterol synthesis and metabolism

Cholesterol synthesis and metabolism are two key regulators for the cholesterol balance in cells [38]. Cholesterol synthesis is regulated by the rate-limiting enzyme HMG-CoA reductase in the endoplasmic reticulum, and SREBP-2 activation leads to the transcription of HMG-CoA reductase mRNA [39]. Based on the observation that GA decreased cellular cholesterol, we examined whether the synthesis of cholesterol was affected by GA. Real-time PCR analysis showed that GA (1 μM) reduced the mRNA levels of HMG-CoA reductase by 31 ± 5% ($n = 3$, $P < 0.05$) and SREBP-2 by 37 ± 5% ($n = 3$, $P < 0.05$) (Fig. 8A), respectively. The mRNA levels of the two molecules decreased significantly. However, the decreased mRNA levels are not enough to make the cholesterol content reduced by 81% when treated with GA (Fig. 6B), which suggested that GA decrease cholesterol content not mainly by blocking its synthesis. Thus, we further investigated the relation between cholesterol metabolism and GA. As shown in Fig. 8B, the mRNA levels of ACAT-1 and ACAT-2 (two key enzymes in cholesterol metabolism) were increased by 76 ± 10% ($n = 3$, $P < 0.01$) and 26 ± 7% ($n = 3$, $P < 0.05$), respectively, when treated with 1 μM GA. These results showed that GA reduced the content of cholesterol in cells via both synthesis and metabolism steps, which contributed to the inhibitory effect of GA on cell adhesion.

## 4. Discussion

It has been known that the majority of early-stage tumors are not life threatening. However, a small amount of primary tumor cells will progress to metastatic tumors, which give rise to a devastating and largely incurable disease and it is major cause of the death. Tumor metastasis is comprised of multiple steps and thus tumor cells are required to express a variety of properties including altered adhesiveness, increased motility and invasive capacity to complete the metastatic process [40]. Interruption of these steps may be a strategy for prevention and treatment of breast cancer metastasis. Anti-metastasis drugs involved in these mechanisms have been extensively investigated [41]. The initial invasive action of metastatic cells involves interaction of tumor cells with the ECM, that is, cell matrix adhesion. Once malignant cells have detached from the primary tumor, they bombard the surrounding basement membrane, and adhere to its meshwork of collagen type IV, laminin, and fibronectin [42]. In this study, we focused on the inhibitory effect of GA on adhesion of human cancer cells based on the anti-metastasis activity reported before. This effect is due to suppressing integrin β1 and membrane lipid rafts, thus inhibiting integrin β1 clustering and the lipid raft-associated integrin signaling pathway. GA (0.25 μM) has little effects in all the experiments, however, the higher concentrations of GA (0.5 μM and 1 μM) have more obvious inhibitory effects on cancer cell adhesion and integrin β1-mediated signaling (Figs. 1B, 4 and 5B), such as p-FAK-Tyr397 and p-c-Src. Especially, 1 μM GA owns significant effects on integrin β1 expression, lipid raft clustering, cholesterol content, as well as cholesterol synthesis and metabolism (Figs. 2, 3, 5, 6 and 8), which sufficiently demonstrated the mechanism involved in.

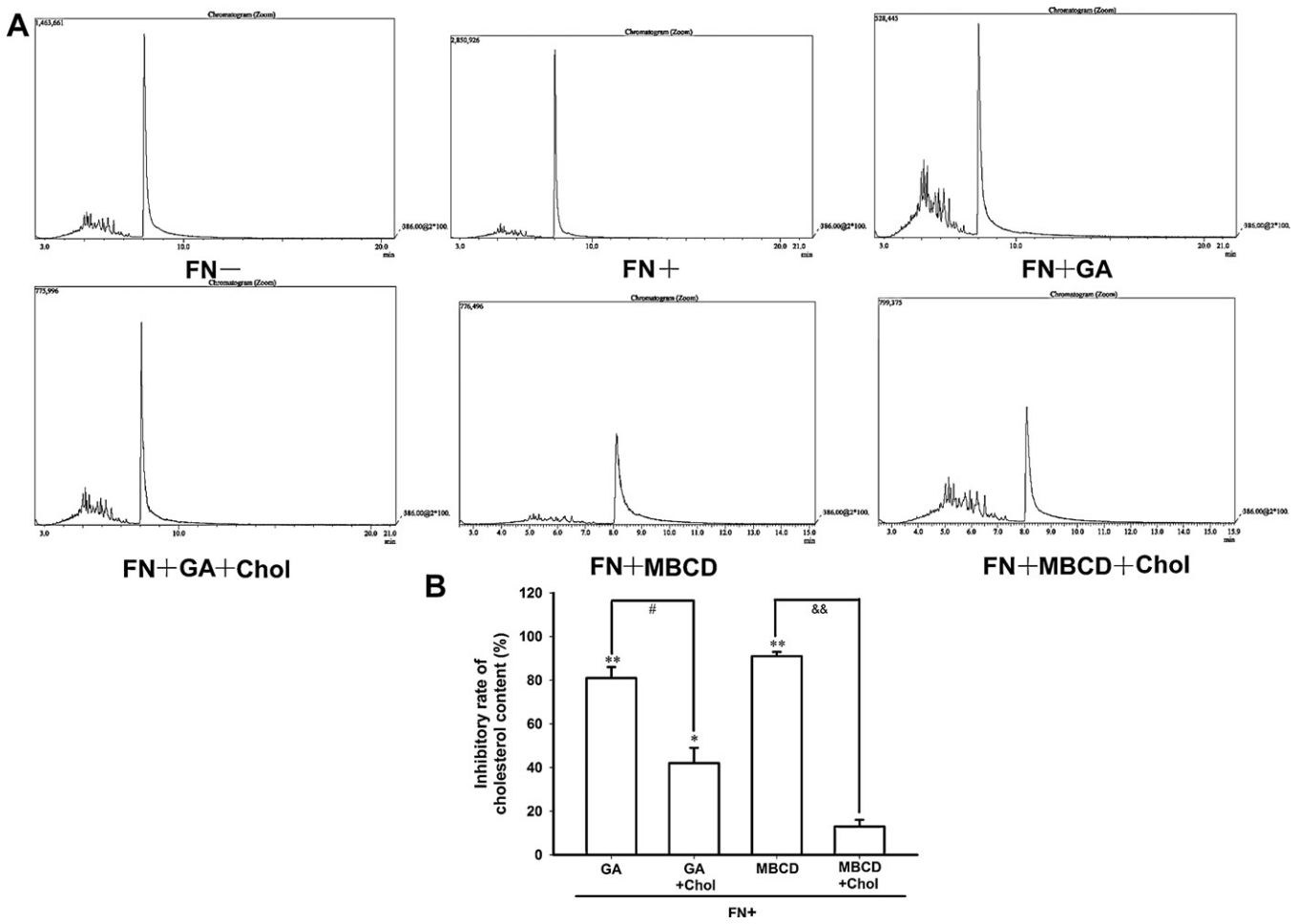


**Fig. 6.** GA reduces the cholesterol content in cells. (A) GC–MS analysis was employed to examine cholesterol content of cells. Chromatograms between 3 min and 21 min (selected ion monitoring *m*/*z* 386) are shown. (B) Histogram indicates the inhibitory rate of cholesterol content. $^{*}P < 0.05$ or $^{**}P < 0.01$ represents significant difference from the fibronectin group. $^{\#}P < 0.05$ represents significant difference between GA group and GA + Chol group. $^{\&\&}P < 0.01$ represents significant difference between MBCD group and MBCD + Chol group.

GA attracted attention as a potential anticancer drug. The relationship between structure and anti-proliferation activity of GA in cancer cells is clear. The topological structure of GA contains two parts: a planar region including rings A, B, and C, as well as a caged core D ring system (Fig. 1A). Preliminary structure-activity relationship studies indicated that it is well tolerated to modify 6-hydroxyl group, 8-ketone group, two isopentenyl groups and 30-carboxyl group in GA, whereas the reduction of 9, 10-double bond of the α, β-unsaturated ketone leads to dramatically decreased potency, demonstrating its essential role in maintenance of the conformation of the 4-oxa-tricyclo [4.3.1.$0^{3,7}$] dec-2-one ring system for anti-proliferation activity [43].

Previous work has shown that GA inhibits the adhesion of MDA-MB-231 cells to matrigel [20]. In the present study, adhesion of MDA-MB-231 cells, HCT-116 cells, and HepG2 cells to fibronectin is suppressed by GA. As shown in Fig. 1B, the different inhibitory effects displayed by GA may attribute to the character of different cancer cell lines. MDA-MB-231 is a high metastatic cell line [44], HCT-116 is a metastatic cell line [45], and HepG2 is a low metastatic cell line [46]. These results indicate that GA might have a better inhibitory effect on adhesion of a high metastatic cell line, which owns more significance for drug anti-metastasis investigation. However, the mechanism why GA has a better effect on the high metastatic cell line needs further studies.

Fibronectin associated integrin signaling plays an important part in cell adhesion [47]. The cell–matrix interaction via fibronectin and its integrin β1 receptor mediates the adhesion of many kinds of cancer cells [48]. Except for the downregulation of integrin (Fig. 3), GA efficiently inhibits the phosphorylation of FAK at Tyr397 as well as c-Src activity, then disrupts FAC formation, thereby inhibits cell adhesion (Fig. 4B and D). Moreover, integrin-ECM protein interactions trigger outside-in and inside-out signaling, which initiate a cascade of events that include the release of proteolytic enzymes (e.g., MMPs). It has been reported that perturbation of various integrins induces upregulation of MMPs, leading to an increase in invasive ability *in vitro* [49]. Our previous study demonstrates that GA inhibits the phosphorylation of ERK1/2 and JNK in MDA-MB-231 cells, which blocks the activation of MMP-2 and MMP-9, and finally results in the anti-invasive effect. It is reported that the MAPK signaling pathway also participates in the invasion mediated by integrin-FAK signaling pathway [50]. These suggest that the inhibitory effect of GA on integrin β1-FAK signaling pathway may also contribute to its anti-invasive activity. These two works is complementary and could be integrated into the investigation of the anti-metastasis effect of GA.

There are several steps involved in integrin-initiated signaling action, including integrin receptor clustering or oligomerization. Recent evidence suggests that integrin clustering and function are regulated by recruitment into discrete cholesterol-rich lipid rafts [51]. The association of integrins with lipid rafts is an important factor in the regulation of integrin activation [13]. One way that rafts transmit signals involves their coalescence into large plat-

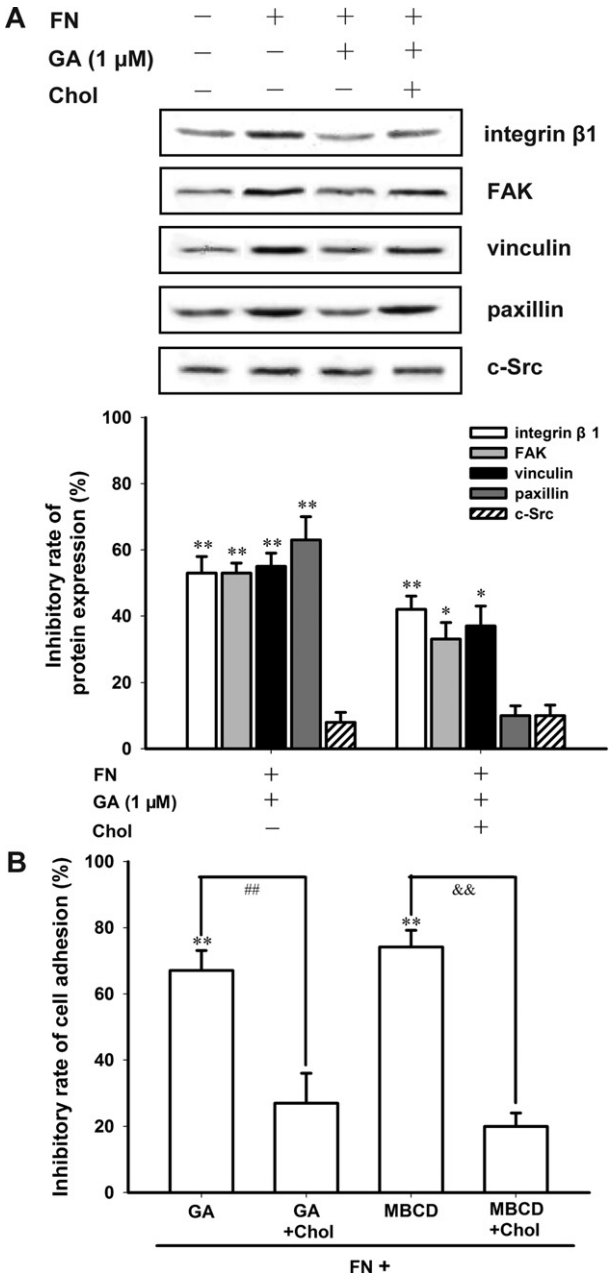


**Fig. 7.** Cholesterol level is involved in GA-mediated inhibition of cell adhesion. (A) Addition of cholesterol partially reverses the effect of GA on translocation of integrin signaling molecules to lipid rafts. Expression of integrin β1 and FAC was detected by Western blotting analysis. $^{*}P < 0.05$ or $^{**}P < 0.01$ represents significant difference from the fibronectin group. (B) Cholesterol replenishment partially blocks the inhibitory effect of GA on cell adhesion. $^{**}P < 0.01$ represents significant difference from the fibronectin group. $^{\#\#}P < 0.01$ represents significant difference between GA group and GA + Chol group. $^{\&\&}P < 0.01$ represents significant difference between MBCD group and MBCD + Chol group.

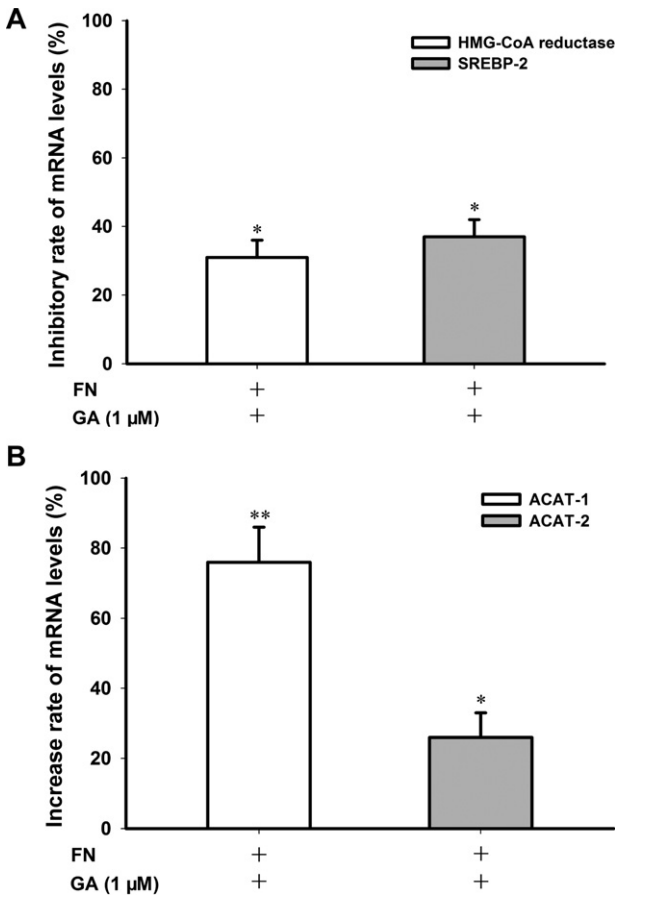


**Fig. 8.** GA regulates cholesterol synthesis and metabolism. (A) GA decreases the mRNA level of HMG-CoA reductase and SREBP-2. (B) GA increases the mRNA level of ACAT-1 and ACAT-2. $^{*}P < 0.05$ or $^{**}P < 0.01$ represents significant difference from the fibronectin group.

forms through which signaling proteins translocate and concentrate [52]. After exposure to fibronectin, small membrane rafts are observed to coalesce into larger patches (Fig. 5A). The newly created larger rafts assemble certain proteins, which facilitate transmembrane signaling [34]. GA disrupts the formation of lipid rafts, as well as suppresses integrin β1 expression and clustering of integrin β1 with rafts patches (Fig. 5A).

Membrane raft structural integrity is required for β1 integrin-mediated mechanotransduction responses [53]. Although the molecular mechanisms of integrin-lipid raft interactions are unclear, cholesterol, the main essential component of lipid rafts, has been reported to play specific roles in regulating integrin functions [54]. Reduction of the cholesterol concentration can lead to the loss of the signaling function of lipid rafts [55]. Our results showed that GA significantly reduces the cholesterol level of cells, consequently suppressing lipid rafts coalescence and cell adhesion (Figs. 5 and 6).

Recent studies have suggested that the membrane lipid profile differs substantially between cancerous and noncancerous cells. Certain cancer cells have relatively high levels of cholesterol and saturated fatty acids, including sphingolipids [56]. Thus, it is meaningful to investigate whether GA could reduce cholesterol content in noncancerous cells. Because cholesterol is essential for many cellular processes, such as energy metabolism and proliferation, GA-induced reduction of cholesterol may result in other biological effects, which requires further studies. Cholesterol balance regulation in cells is an extremely complex process. Cholesterol is obtained through both exogenous uptake and endogenous synthesis and can be released/metabolized via other interrelated processes [57]. Cholesterol synthesis is mainly regulated by HMG-CoA reductase and SREBP-2 [39]. Other mechanisms that control cholesterol homeostasis include the exogenous uptake of cholesterol via low density lipoprotein receptor (LDL-r) and scavenger receptor class B type I (SR-BI) in clatherin-coated pits [58]. Free cholesterol is esterified by ACAT-1 and ACAT-2 [59]. In our work, GA disturbs cholesterol homeostasis, and regulates cholesterol synthesis and metabolism (Fig. 8), which contributes to a reduction of cholesterol (Fig. 6). It is just a possible mechanism involved in, and whether GA influences exogenous uptake of cholesterol needs further studies.

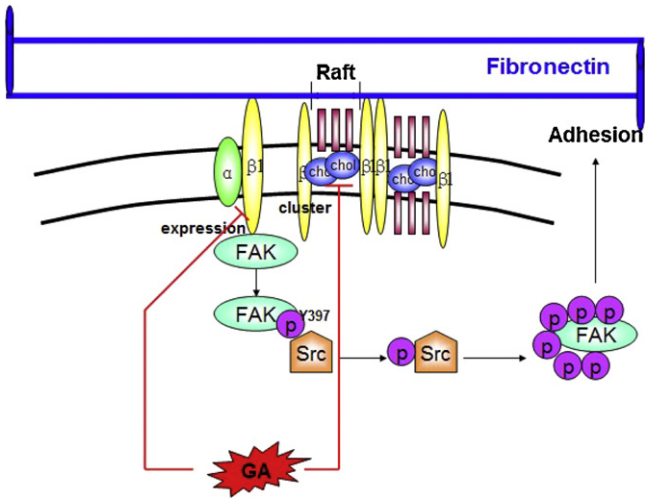


**Fig. 9.** A possible mechanism underlying the inhibitory effect of GA on adhesion.

Taken together, we present the evidence that GA significantly inhibits cancer cell adhesion by downregulation of integrin β1 and cholesterol levels, which suppresses the lipid raft-associated integrin signaling pathway (Fig. 9). As such, our results provide a new understanding of the anti-metastatic activity of GA, and the underlying molecular mechanism of GA anti-cancer effects merit further investigations.

## Conflict of interest

The authors have declared no conflict of interest.

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.09.013.

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