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Gallic Acid Induces G2/M Phase Arrest of Breast Cancer Cell MCF-7 through Stabilization of $p27^{Kip1}$ Attributed to Disruption of $p27^{Kip1}$/Skp2 Complex

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ABSTRACT: Gallic acid (GA), 3,4,5-trihydroxybenzoic acid, is a natural polyphenolic acid and widely found in gallnuts, tea leaves and various fruits. Previous studies have shown that GA possesses anti-inflammatory, antiallergic and anticarcinogenic activity. In the present study, we aim to investigate the antitumor effects of GA on breast cancer cell. Our results revealed that GA treatment significantly reduced the cell growth of human breast cancer cell MCF-7 in a dose-dependent manner. Further flow cytometric analysis showed that GA induced significant G2/M phase arrest but slightly affected the population of sub-G1MCF-7 cells. Therefore, levels of cyclins, cyclin-dependent kinases (CDKs), and their regulatory proteins involved in S-G2/M transition were investigated. Our findings revealed that levels of cyclin A, CDK2, cyclin B1 and cdc2/CDK1 were diminished; in contrast, levels of the negative regulators $p27^{Kip1}$ and $p21^{Cip1}$ were increased by GA treatment. Additionally, Skp2, a specific ubiquitin E3 ligase for polyubiquitination of $p27^{Kip1}$ was reduced by GA treatment. Further investigation showed that GA attenuated Skp2-$p27^{Kip1}$ association and diminished polyubiquitination of $p27^{Kip1}$ in MCF-7 cells. Moreover, knockdown of $p27^{Kip1}$ but not $p21^{Cip1}$ significantly alleviated GA-induced accumulation of G2/M phase. These findings indicate that GA may upregulate $p27^{Kip1}$ level via disruption of $p27^{Kip1}$/Skp2 association and the consequent degradation of $p27^{Kip1}$ by proteosome, leading to G2/M phase arrest of MCF-7 cell. It is suggested that GA should be beneficial to treatment of breast cancer and $p27^{Kip1}$-deficient carcinomas.

KEYWORDS: gallic acid, cell cycle arrest, MCF-7, $p27^{Kip1}$, $p21^{Cip1}$, Skp2

INTRODUCTION

Gallic acid (GA), known as 3,4,5-trihydroxylbenzoic acid, is a polyhydroxyl phenolic compound and is ubiquitously present in fruits, gallnuts, green tea and red wine.[1−5] GA has been demonstrated to have a broad spectrum of biological activities, including antimicrobial,[6−8] and anti-inflammatory[9] activities. GA has been investigated as a potential anticancer agent in various human cancer cell lines, such as TE-2 (esophageal cancer), MKN-28 (gastric cancer), HT-29 and Colo201 (colon cancer), MCF-7 (breast cancer) and CaSki (cervix cancer).[10] Additionally, previous studies have shown that GA induced apoptosis in cancer cells in association with oxidative stresses derived from reactive oxygen species (ROS), mitochondrial dysfunction and an increase in intracellular Ca^{2+} level.[11,12] However, GA also exhibits antiapoptotic potential in normal human lymphocytes[13] and acts as strong natural antioxidant scavenging ROS.[13−16] Molecular mechanisms underlying induction of GA in the aspect of cell fate remains sketchy.

Breast cancer is the commonest cancer in most countries in Asia, and the incidence is increasing at a more rapid rate than in Western countries, which may be due to changes in the lifestyle and diet.[17] Hormone replacement therapy such as prolonged exposure to estrogen and/or progesterone and reproductive history are the most major risk factors for breast cancer.[18] Additionally, the importance of estrogen in breast cancer development is also supported by studies demonstrating the occurrence of marked changes in estrogen signaling and in the expression of the two estrogen receptors (ERs), ER alpha and ER beta, during breast tumorigenesis and progression.[19−21] To date, a universal surgical resection followed by radiotherapy and/or chemotherapy are the most common treatments for breast cancer to prevent its metastasis and recurrence.[22] Therefore, the means that intervenes in the proliferation and progression of breast cancer is important prior to the clinical treatments.

The significance of cell cycle mediators involved in carcinogenesis is now well documented. Critical genes that regulate cell cycle checkpoints have been demonstrated to be lost and/or aberrant in a variety of cancers in human.[23] Cells progress through the various phases of the cell cycle through the interactions of different cyclins with their specific kinases, cyclin-dependent kinases (CDKs),[24] which can be negatively regulated by two classes of CDK inhibitors, inhibitors of CDK4 (INK4) and kinase inhibitor proteins (KIPs). The latter include $p21^{Cip1}$,[25] $p27^{Kip1}$ [26] and $p57^{Kip2}$.[27,28]

In the present study, we focused on the effects of GA on cell proliferation and the cell cycle of breast cancer cells with emphasis on the underlying mechanism. To investigate the alteration of cell proliferation and cell cycle distribution induced by GA, MTT assay and flow cytometric analysis were performed. The expression level of important cell cycle regulators was determined by immunoblotting. Additionally, the protein−protein interactions between the regulators and GA were also demonstrated by immunoprecipitation and immunoblotting.

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MATERIALS AND METHODS

Chemicals. 2-Propanol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 1-butanol, dimethyl sulfoxide (DMSO), deoxycholic acid, dithiothreitol, EDTA, glycerol, Igepal CA-630, phenylmethylsulfonyl fluoride (PMSF), sodium chloride (NaCl), potassium chloride (KCl), sodium dodecyl sulfate (SDS), sodium phosphate, Tris-HCl and trypsin/EDTA were purchased from Sigma (St. Louis, MO). Flavonoids, including caffeic acid, catechin, chlorogenic acid, coumaric acid, ellagic acid, epicatechin, ferulic acid, GA, gossypin, hydroxyflavin, naringenin, protocatechuic acid, quercetin, resveratrol and rutin, were obtained from Sigma and Extrasynthese (Genay, France). Antibodies against cyclin A, cyclin B1, cdc2/CDK1, CDK2, $p21^{Cip1}$, $p27^{Kip1}$, S-phase kinase-associated protein 2 (Skp2) and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-conjugated antibodies against mouse IgG or rabbit IgG were purchased from Cell Signaling Technology (Beverly, MA).

Cell Culture and GA Treatments. The breast cancer cell line MCF-7 was obtained from American Type Culture Collection (ATCC; Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v fetal bovine serum, 1% nonessential amino acid, 1% L-glutamine (Gibco BRL, Gaithersburg, MD) and 100 μg/mL penicillin/streptomycin (Sigma) at 37 °C in a humidified atmosphere with 5% CO_2. Cells were seeded in 6-well culture plates at an initial density of 2×10^5 cells/mL and grown to approximately 80% confluence. For GA treatments, cells were starved for 16 h (h) in serum-free DMEM and then treated with different concentrations of GA in DMEM (1, 5, and 10 μg/mL for cell proliferation; 2, 4, 6, 8, 10, and 12 μg/mL for flow cytometric analysis; 2, 4, and 6 μg/mL for immunoblotting) for 24 h. After treatments, the cells were washed with phosphate-buffered saline (PBS; 25 mM sodium phosphate, 150 mM NaCl, pH 7.2) and then collected by centrifugation (800g × 5 min) for subsequent analyses.

Cell Growth Assay. Cell growth was determined by MTT assay. Cells were seeded at a density of 4×10^4 cells/well in a 24-well plate and cultured for 24 h. Then, the cells were treated with GA at various concentrations (0, 1, 5, and 10 μg/mL) for 24 h. Each concentration was performed in triplicate. After the treatments, the medium was aspirated, followed by washing the cells with PBS. Then, the cells were incubated with MTT solution (5 mg/mL)/well for 4 h. The supernatant was removed, and formazan was solubilized in isopropanol and measured spectrophotometrically at 563 nm. The percentage of viable cells was estimated by comparing with untreated cells.

Flow Cytometric Analysis. Cells synchronized at G0 phase by serum starvation for 24 h were incubated in fresh serum-containing medium to allow cell-cycle progression. At various time points after release from G0 arrest, cells were analyzed by flow cytometry to determine cell-cycle distribution. At the end of GA treatment, cells were collected, fixed with 1 mL of ice-cold 70% ethanol, incubated at −20 °C for at least 24 h, and centrifuged at 380g for 5 min at room temperature. Cell pellets were treated with l mL of cold staining solution containing 20 μg/mL propidium iodide (PI), 20 μg/mL RNase A, and 1% Triton X-100 and incubated for 15 min in dark at room temperature. Subsequently, the samples were analyzed in a FACS Calibur system (version 2.0, BD Biosciences, Franklin Lakes, NJ) using CellQuest software. Results were representative of at least three independent experiments.

Protein Extraction. After GA treatments, MCF-7 cells were harvested by using trypsin/EDTA and homogenized in ice-cold lysis buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Igepal CA-630, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM dithiothreitol, 0.1 mM EDTA, 1 mM PMSF]. After sonication at 4 °C for 30 min, the homogenate was centrifuged at 14000g for 10 min, and then the supernatant was transferred into a new 1.5 mL eppendorf and stored at −70 °C for subsequent analysis. Protein concentration was quantitated by Bradford method (protein assay reagent; Bio-Rad Laboratory, Hercules, CA) according to manufacturer's instruction.

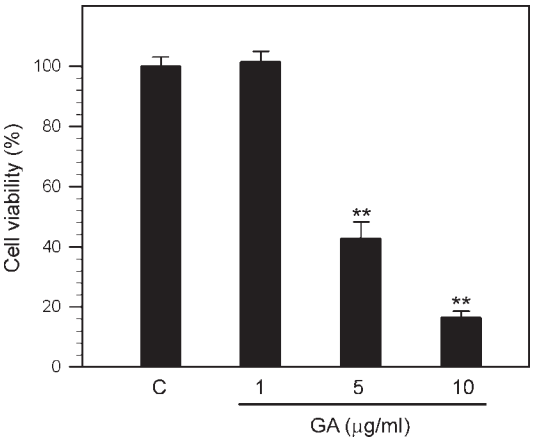

Figure 1. GA suppressed the growth of MCF-7 cells. Cells were treated with indicated concentration of GA for 24 h, and the cell number was analyzed by MTT assay. Data were shown as the means ± SD. Three independent experiments were performed for statistical analysis. **, $p < 0.005$ compared with control (C).

Immunoblot. After GA treatment, aliquots of cell lysate (50 μg of protein) were subjected to a 12.5% SDS−polyacrylamide gel and transferred onto a nitrocellulose membrane as previously described.[32] The blot was subsequently incubated with 5% nonfat milk in PBS for 1 h, probed with a primary antibody against cyclin A, cyclin B1, cdc2/CDK1, CDK2, $p21^{Cip1}$, $p27^{Kip1}$, Skp2 or β-actin for 2 h, and then reacted with an appropriate peroxidase-conjugated secondary antibody for 1 h. All incubations were carried out at 30 °C, and intensive PBS washing was performed between each incubation. After the final PBS wash, the signal was developed by ECL chemiluminescence, and the relative photographic density was quantitated by image analysis system (Fuji Film, Tokyo, Japan).

Immunoprecipitation. Five hundred micrograms of cell lysate protein was precleared with protein-A-conjugated Sepharose beads (Amersham Bioscience, Piscataway, NJ) and immunoprecipitated using monoclonal anti-$p27^{Kip1}$ antibodies. Immune complexes were harvested with protein-A-conjugated Sepharose beads. Immunoprecipitated proteins were subjected to the immunoblots using antibodies against $p27^{Kip1}$ and Skp2.

Specific siRNA Downregulation of $p21^{Cip1}$ and $p27^{Kip1}$. $p21^{Cip1}$ and $p27^{Kip1}$ gene expression was downregulated in bronchial epithelial cells by the use of small-interfering RNA (siRNA). Cells were seeded in 24-well plates or 5×10^4/well and transfected with siRNA with a specific target sequence for human $p21^{Cip1}$, $p27^{Kip1}$ or with nontargeting control oligonucleotides (Thermo Scientific, Dharmacon Products, Lafayette, CO). Cells were transfected with 2 μg of siRNA purified and annealed synthetic oligonucleotides (Thermo Scientific Dharmacon) in a final concentration of 50 nM by the high-efficiency transfection method using DharmaFECT (Thermo Scientific Dharmacon). Eight h after transfection, medium was refreshed and cells were incubated with 10 μg/mL GA in DMEM for 24 h.

Statistical Analysis. Data were expressed as means ± SD of the three independent experiments. Statistical significance analysis was determined by using 1-way ANOVA followed by Dunnett for multiple comparisons with the control. The differences were considered significant for p values less than 0.05.

RESULTS AND DISCUSSION

GA Suppressed Cell Growth of MCF-7 Cells. To investigate the cytotoxic effects of common flavonoids on human breast cancer cells, MCF-7 cells were treated with a series of flavonoids,

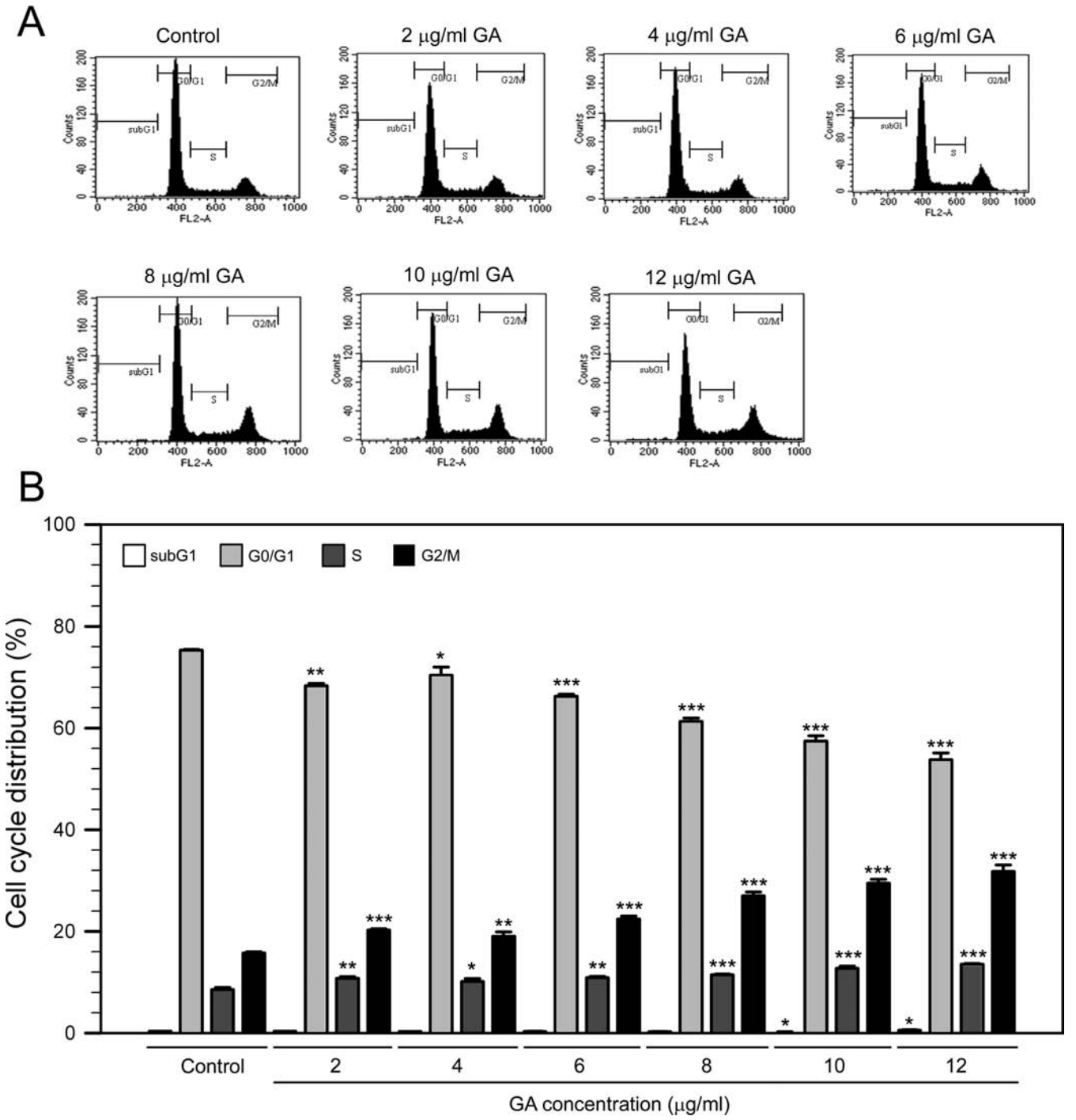

Figure 2. GA induced G2/M phase arrest of MCF-7 cells. (A) Representative cell cycle distribution of MCF-7 treated with a series concentration of GA for 24 h was shown. (B) Ratios of various cell cycle phases, including G0/G1, sub-G1, S and G2/M, were presented. Data were shown as the means ± SD. Three independent experiments were performed for statistical analysis. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$ as compared to corresponding control.

including caffeic acid, catechin, chlorogenic acid, coumaric acid, ellagic acid, epicatechin, ferulic acid, GA, gossypin, hydroxyflavin, naringenin, protocatechuic acid, quercetin, resveratrol and rutin, at different concentrations (1, 5, and 10 μg/mL) for 24 h, and then the cell number was determined. Our results revealed that only GA remarkably inhibited cell proliferation of MCF-7 among the tested flavonoids (data not shown). The cell proliferation of MCF-7 inhibited by GA was shown in Figure 1. GA at higher tested concentration (5 and 10 μg/mL) significantly reduced the cell proliferation of MCF-7 to 42.73 ± 6.18% and 16.56 ± 1.84% of control respectively ($p < 0.005$), whereas the low tested concentration (1 μg/mL) showed no significant influence on cell proliferation ($p = 0.606$).

Inhibited cell proliferation can result from various cell death and/or cell cycle arrest. Recent studies have shown that high concentration of GA (17 - 136 μg/mL) significantly inhibits the growth of HeLa cervical cancer cell and A549 lung carcinoma cell through apoptosis and/or necrosis.[33,34] Our results show that

GA significantly inhibited the cell growth of MCF-7 cells by relatively low tested concentrations (2 − 8 μg/mL), Additionally, sub-G1 phase of MCF-7 cells treated with higher concentrations of GA (10 and 12 μg/mL) was slightly increased (<1%, $p < 0.05$ as compared to control). These findings indicate that breast cancer cells are relatively sensitive to GA compared with cervical cancer cells.

GA Induced G2/M Phase Arrest in MCF-7 Cells. Observing suppressed cell proliferation of MCF-7, cell cycle distribution of MCF-7 cell treated with GA was consequently analyzed to investigate the underlying mechanism. After exposure to a series concentration of GA (2, 4, 6, 8, 10, and 12 μg/mL) for 24 h, distribution of population of MCF-7 cells to different cell cycle phases was analyzed and quantitated using flow cytometry. As shown in Figures 2A and 2B, percentage of cells in sub-G1 phase, ranging from 0.36 ± 0.03% to 0.58 ± 0.08%, was not significantly influenced by the GA treatments (2−10 μg/mL). However, ratios of S and G2/M phase, ranging from 8.58 ± 0.29% to 13.54 ± 0.29% and from 15.76 ± 0.26% to 31.81 ± 1.76%, were significantly increased in the presence of GA (2−12 μg/mL). Additionally, ratios of G0/G1 phase, ranging from 75.38 ± 0.12% to 53.79 ± 1.52%, were significantly decreased with GA concentrations. Together, these results revealed that GA treatments increased the ratios of S phase and G2/M phase but decreased the number of G0/G1MCF-7 cells in a dose-dependent manner. Moreover, with 12 μg/mL GA treatment, the ratio of G2/M phase was increased 2.01-fold compared to the control, which was the most altered phase among the four tested cell cycle phases in the MCF-7 cell. These findings revealed that GA induced significant G2/M arrest of MCF-7 cells.

Recently, GA has been reported for its ability to induce apoptosis involved G2/M arrest in human NCI-H460 non-small-cell lung cancer cell.[35] Additionally, digalloylresveratrol, a GA derivative, is also found to inhibit the transition from S to G2/M phase of the cell cycle in human HT-29 colon cancer cell.[36] Interestingly, our findings revealed that GA (12 μg/mL) inhibited cell growth to approximately 20% of control but slightly increased the percentage of subG1 phase (<1%), suggesting that GA may induce not only cell cycle arrest but also autophage and/or cell damage synergistically to inhibit the growth of MCF-7 cells. Taken together, these findings indicate that GA effectively suppresses the growth of MCF-7 cells, which may be attributed to induction of G2/M phase arrest but not apoptosis. The difference in the mechanisms of the anticancer effect of GA concluded in previous studies may be attributed to the various carcinogenesis and the specific cell characteristics for the individual cancer cells.

GA Diminished Protein Levels of Cyclins and CDKs in MCF-7 Cells. Since GA induced G2/M phase accumulation of MCF-7 cells, the effects of GA treatments on protein levels of cyclins and CDKs, the important regulators mediating cell cycle progress, were further investigated. Protein levels of cyclin B1, cdc2/CDK1, cyclin A and CDK2 were determined by immunoblotting and relatively quantitated by densitometric analysis. Our results showed that GA treatments (2−6 μg/mL) dose-dependently decreased the protein levels of cyclin A, CDK2, cyclin B1 and cdc2/CDK1 (Figure 3). With 6 μg/mL GA treatment, the mean levels of cyclin A, CDK2, cyclin B1 and cdc2/CDK1 were reduced to 79.2%, 54.6%, 82.5% and 53.1% of control respectively by densitometric quantitation ($p < 0.05$ as compared to control, C) (Figure 3).

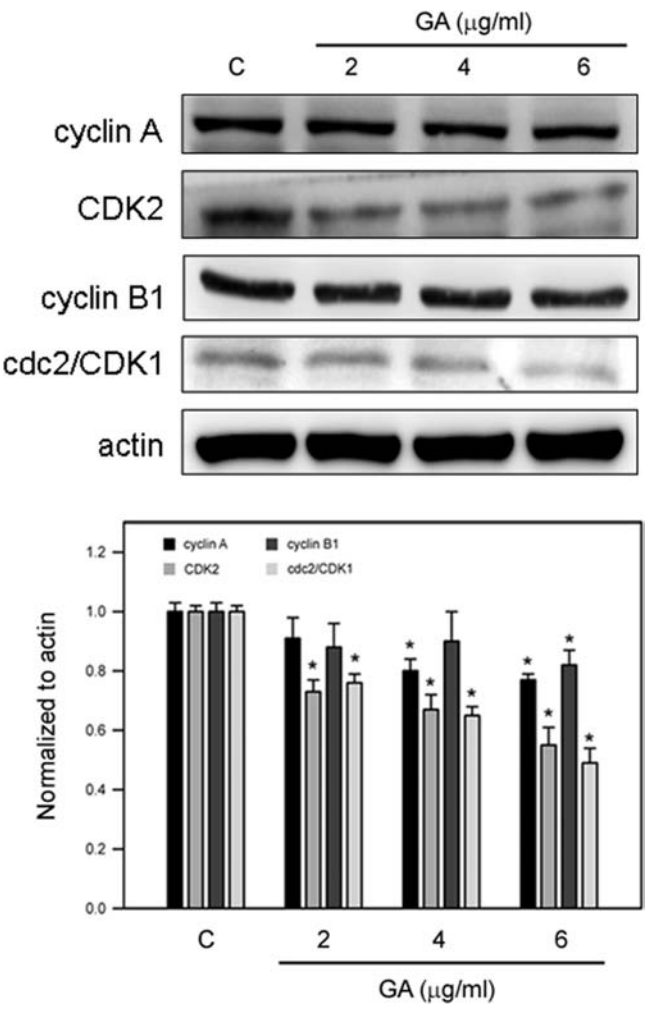

Figure 3. GA reduced protein levels of cell cycle regulators, cyclin A, CDK2, cyclin B1 and cdc2/CDK1, in MCF-7 cells. Cells were treated with the indicated concentration of GA for 24 h, and then the cell lysates containing 30 μg of protein were subjected to immunoblot for detection of the indicated proteins. Protein levels were relatively quantitated by densitometric analysis using level of actin as control. Quantitative data ($n = 3$) were acquired by densitometric analysis using actin as control and are presented as the means ± SD. *, $p < 0.05$ as compared to corresponding control.

The originally identified A-type cyclin, also known as cyclin A2, is ubiquitously expressed in mitotically dividing cells and is upregulated in a variety of cancers.[37,38] In late G1 phase, cyclin A binds to CDK2 to promote transition to S phase and plays important roles in replication of DNA and centromere in S phase.[39] The roles of the different B-type cyclins are not fully understood; however, in general, the B-type cyclins appear during the G2−M phase transition of the cell cycle. During the G2−M phase transition, cyclin B1 binds to cdc2/CDK1 to form mitosis-promoting factor, which facilitates the transition from G2 to M phase of the cell cycle.[40] Therefore, reduced levels of cyclin A and cyclin B1 attenuate the activation of both cdc2/CDK1 and CDK2, which consequently leads to the cell cycle arrest at S phase and G2/M phase. These findings are consistent with our flow cytometric analysis that presents a significant increased ratio of S phase and G2/M phase in GA-treated MCF-7 cells (Figure 2), suggesting that GA may trigger the G2/M cell cycle arrest via downreglation of cyclin A, CDK2, cyclin B1 and cdc2/CDK1.

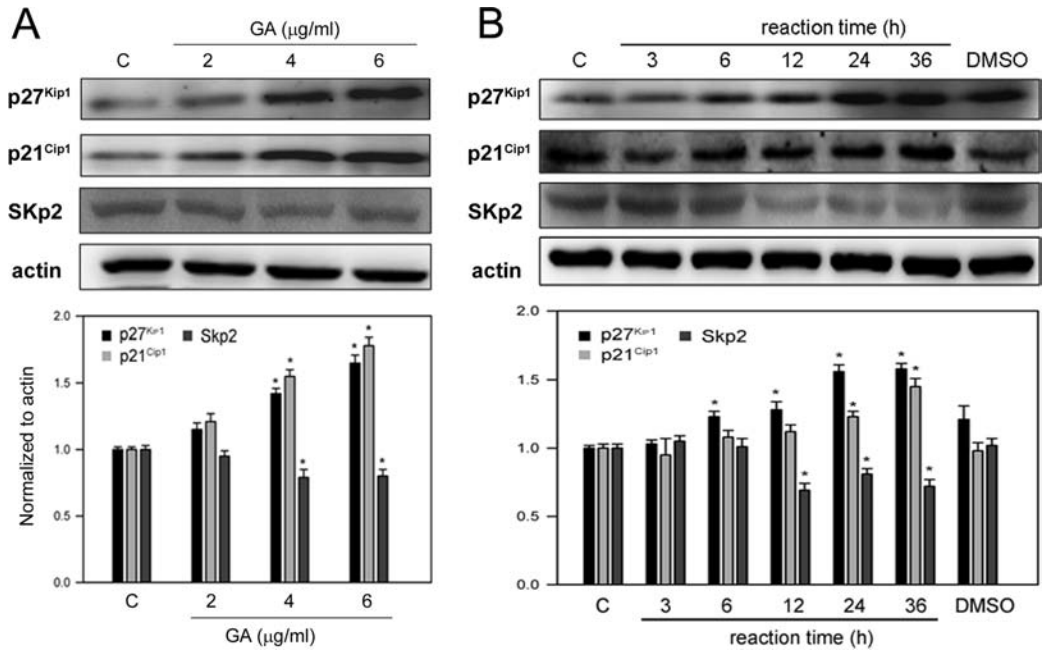

Figure 4. GA diminished protein levels of $p27^{Kip1}$, $p21^{Cip1}$ and Skp2 in MCF-7 cells. Cells were treated with indicated concentration of GA for 24 h (A) or treated with 10 μg/mL GA for indicated time (B), and then the cell lysates containing 30 μg of protein were subjected to immunoblot for detection of indicated proteins. Quantitative data ($n = 3$) were acquired by densitometric analysis using actin as control and presented as the means ± SD. *, $p < 0.05$ as compared to corresponding control.

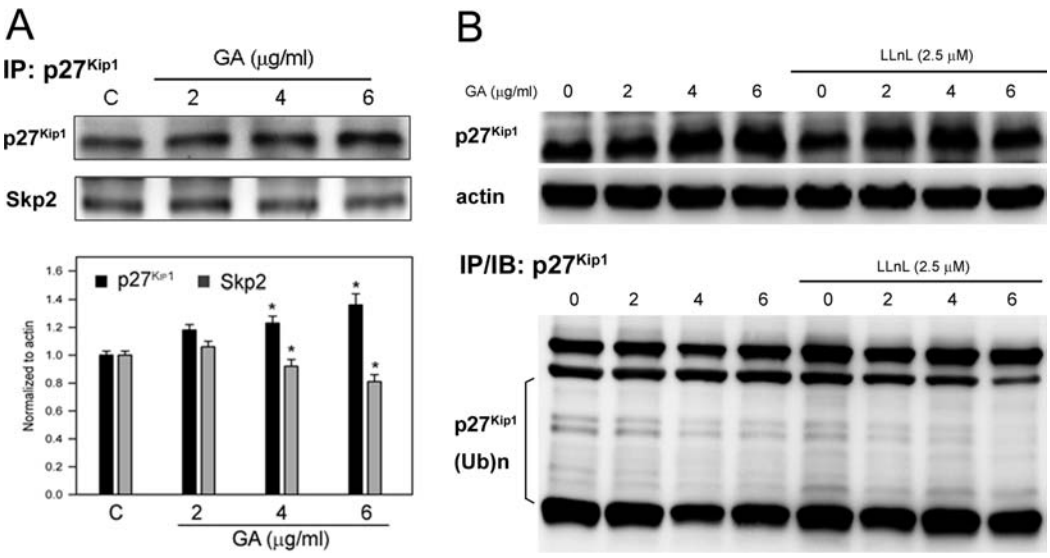

Figure 5. GA increased $p27^{Kip1}$ levels via diminishing association of $p27^{Kip1}$/Skp2 and polyubiquitination of $p27^{Kip1}$ in MCF-7 cells. (A) Cells were treated with 2, 4, and 6 μg/mL GA for 24 h, and then the cell lysates were immunoprecipitated with anti-$p27^{Kip1}$ antibodies. The immunoprecipitated proteins were subjected to immunoblot for detection of $p27^{Kip1}$ and Skp2. Quantitative data ($n = 3$) were acquired by densitometric analysis using actin as control and presented as the means ± SD. *, $p < 0.05$ as compared to corresponding control. (B) Cells were treated with a series concentration of GA (2, 4, and 6 μg/mL) or cotreated with the series concentration of GA and proteosome inhibitor LLnL (2.5 μM) for 24 h. The cell lysates were subjected to immunoblot for detection of $p27^{Kip1}$ (upper panel) or were immunoprecipitated with anti-$p27^{Kip1}$ antibodies, and then the immunoprecipitated proteins were subjected to immunoblot for detection of $p27^{Kip1}$ (lower panel).

GA Altered Protein Levels of Cell Cycle Regulators in MCF-7 Cells. Because levels of cell cycle mediators, including cyclin A, CDK2, cyclin B1 and cdc2/CDK1, were found diminished by GA treatments, the effects of GA treatments on CDK inhibitors, $p27^{Kip1}$ and $p21^{Cip1}$, were further investigated. As shown in Figure 4A, GA treatments dose-dependently increased mean levels of $p27^{Kip1}$ and $p21^{Cip1}$ up to 1.68- and 1.76-fold as compared to control. The ubiquitin−proteasome system is known to control the abundance of short-lived regulatory proteins such as p53, c-Myc, IκBα, β-catenin, $p27^{Kip1}$, and cyclins.[41] Polyubiquitin conjugation of lysine residues in proteins as a result of collaboration with a ubiquitin activating enzyme (E1), a ubiquitin-conjugation enzyme (E2), and a ubiquitin

ligase (E3) is required for selective recognition and degradation by the 26S proteasome.[42] Additionally, E3 ubiquitin ligases are often reported to be involved in carcinogenesis or tumor development.[41] Skp2, belonging to SCF-type E3 ligase, can ubiquitylate various cancer-associated proteins, including $p27^{Kip1}$, $p21^{Cip1}$, $p57^{Kip2}$, and c-Myc.[43−45] Therefore, effects of GA on Skp2 were further investigated. As shown in Figure 4, levels of Skp2 were decreased in MCF-7 cells treated with GA in a dose-dependent manner. Treating with the highest GA concentration (6 μg/mL), the level of Skp2 was diminished to 81.6% of the control.

The changes in levels of $p27^{Kip1}$, $p21^{Cip1}$ and Skp2 in MCF-7 cells treated with GA (4 μg/mL) for several time periods (3−36 h) were also determined. Our results revealed that $p27^{Kip1}$ and $p21^{Cip1}$ levels were increased in a time-dependent manner (3−24 h), and in contrast, Skp2 level was decreased with the reaction times (0−12 h) in MCF-7 cells treated with GA (Figure 4B). Both mean levels of $p27^{Kip1}$ and $p21^{Cip1}$ reached to a maximum 1.58-fold and 1.45-fold increase of control at 36 h of GA treatment. Interestingly, mean level of Skp2 reached the minimal mean level (72.8% of control) at 12 h, slightly increased to 81.6% of control at 24 h, and then decreased to 78.4% of control at 36 h of GA treatment (Figure 4B). Taken together, these results indicate that GA is able to increase the level of $p27^{Kip1}$ and $p21^{Cip1}$, and to decrease the level of Skp2 simultaneously. It is suggested that GA-elevated $p27^{Kip1}$ and $p21^{Cip1}$ level may result from the suppression of the negative regulatory protein Skp2, which subsequently leads to the cell cycle arrest at G2/M phase.

GA Attenuated the Interaction of $p27^{Kip1}$ and Skp2 and the Polyubiquitination of $p27^{Kip1}$ in MCF-7 Cells. GA treatment was found to enhance $p27^{Kip1}$ level and diminish Skp2 level in MCF-7 cells; therefore, effects of GA on interaction of $p27^{Kip1}$ and Skp2 were further investigated. As shown in Figure 5A, GA treatment significantly reduced abundance of $p27^{Kip1}$-associated Skp2 and increased the level of $p27^{Kip1}$ in the $p27^{Kip1}$/Skp2 complex. Additionally, the increase of $p27^{Kip1}$ by GA treatment was similar to that by GA and proteasome inhibitor (LLnL) cotreatment in MCF-7 cells (Figure 5B). Accordingly, the effects of GA on polyubiquitination of $p27^{Kip1}$ were also determined. Our results showed that GA diminished polyubiquitination of $p27^{Kip1}$, and that the attenuation of polyubiquitination by GA and by GA combining proteasome inhibitor LLnL was similar (Figure 5B). These findings indicate that GA significantly attenuates the binding of Skp2 to $p27^{Kip1}$ and the polyubiquitination of $p27^{Kip1}$, suggesting that GA may disrupt the interaction of Skp2 and $p27^{Kip1}$ to diminish the polyubiquitination of $p27^{Kip1}$ by Skp2, and then increase $p27^{Kip1}$ level to cause G2/M phase arrest.

It has been reported that Skp2 recognizes Thr187-phosphorylated $p27^{Kip1}$ in collaboration with cdc kinase subunit 1 to promote polyubiquitination of $p27^{Kip1}$.[43] However, whether GA diminishes Thr187-phosphorylation of $p27^{Kip1}$ and subsequently attenuates Skp2-$p27^{Kip1}$ association in MCF-7 cell needs further investigation. Recent studies have shown that alteration in the expression of $p27^{Kip1}$ in human malignancies may lead to uncontrolled cellular proliferation and adversely affect clinical outcome.[46−50] Thus, low levels of $p27^{Kip1}$ were found to profoundly impact tumor progression and accurately predict poor prognosis in various cancers, including breast, colorectal, and prostate carcinomas as well as other epithelial carcinomas, sarcomas, and hematologic malignancies.[46−51] Taken together, these studies have suggested that loss of $p27^{Kip1}$ may contribute to uncontrolled tumor proliferation. Our findings indicate that GA can attenuate the interaction of $p27^{Kip1}$ and Skp2 to diminish polyubiquitination of $p27^{Kip1}$ and then restore the level of $p27^{Kip1}$, suggesting that GA should be beneficial to the uncontrolled tumor proliferation attributed to loss of $p27^{Kip1}$.

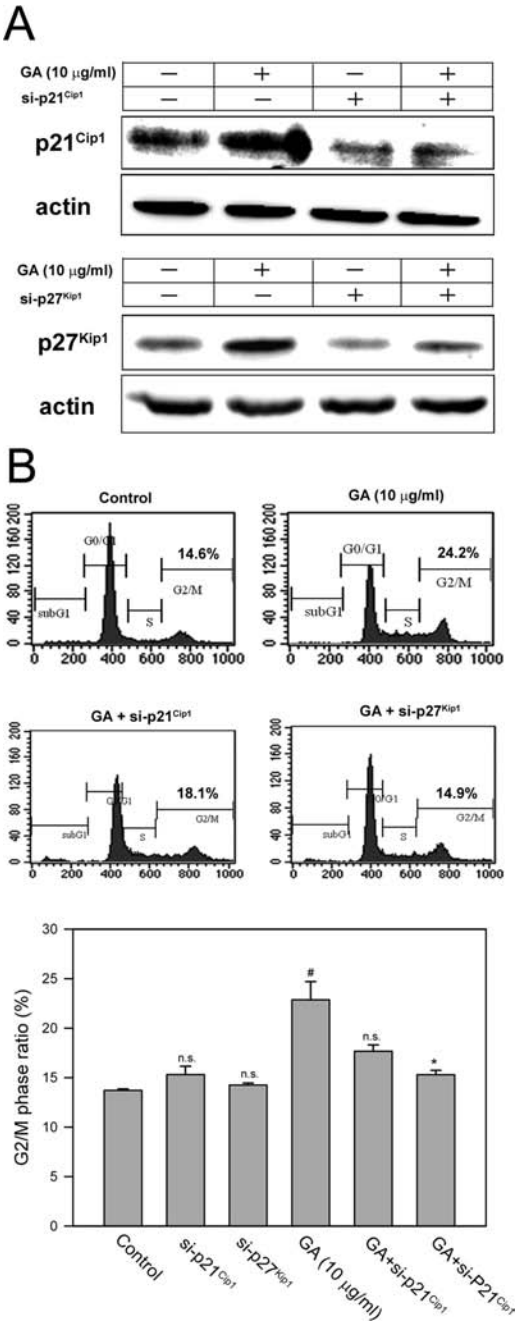

Figure 6. Downregulation of $p21^{Cip1}$ and $p27^{Kip1}$ alleviated GA-induced accumulation of G2/M phase in MCF-7 cells. Cells were pretreated with siRNA for $p21^{Cip1}$ (si-$p21^{Cip1}$) and $p27^{Kip1}$ (si-$p27^{Kip1}$), and then treated with 10 μg/mL GA for 24 h. (A) The treated cells were lysed, and the crude proteins were subjected for immunoblot for detection of $p21^{Cip1}$ or $p27^{Kip1}$. (B) The treated cells were analyzed for cell cycle distribution by flow cytometry. Quantitative data (n = 3) were presented as the means ± SD; n.s., not significant; *, $p < 0.05$ as compared to GA alone treatment.

Downregulation of $p27^{Kip1}$ but Not $p21^{Cip1}$ Significantly Reduced GA-Induced G2/M Phase Arrest of MCF-7 Cells. To further investigate roles of $p21^{Cip1}$ and $p27^{Kip1}$ in GA-induced G2/M phase arrest of MCF-7 cells, downregulation of $p21^{Cip1}$ and $p27^{Kip1}$ by specific siRNA was performed. As shown in

Figure 6A, treatment of siRNA for $p21^{Cip1}$ and $p27^{Kip1}$ significantly decreased their protein levels induced by GA (10 μg/mL) respectively. Additionally, analysis of G2/M cell cycle distribution revealed that GA treatment increased mean G2/M phase to 22.9%, which was diminished to 17.7% by pretreatment of siRNA for $p21^{Cip1}$ (si-$p21^{Cip1}$, $p = 0.0641$) and to 15.3% by pretreatment of siRNA for $p27^{Kip1}$ (si-$p27^{Kip1}$, $p = 0.0301$) (Figure 6B).

It has been reported that overexpression of $p21^{Cip1}$ inhibits proliferation of mammalian cells, and all cyclin−CDK complexes, indicating that it is a universal cyclin−CDK inhibitor.[29] In contrast to $p21^{Cip1}$, $p27^{Kip1}$ is a negative regulator of protein kinases for cyclin E-CDK2 and cyclin A-CDK2 which drive cells into the S phase of the cell division cycle.[30] In addition, $p27^{Kip1}$ has been reported to play important roles in G2/M checkpoint as tumor suppressor.[31] Our findings show that downregulation of $p27^{Kip1}$ but not $p21^{Cip1}$ significantly alleviates G2/M phase accumulation induced by GA, implying that $p27^{Kip1}$ might play a more important role than $p21^{Cip1}$ dose in GA-induced G2/M phase arrest of MCF-7 cells. However, further mechanism of $p21^{Cip1}$ and $p27^{Kip1}$ and the interplay between both cell cycle regulators in the GA-induced G2/M phase arrest needs more investigation.

In conclusion, the present study demonstrates that GA significantly reduces cell proliferation of human breast cancer cell MCF-7 via G2/M phase arrest, which may result from the increase of $p27^{Kip1}$ and $p21^{Cip1}$ by suppressing Skp2-mediated polyubiquitination. These findings indicate that GA may provide potent therapeutic effects against breast cancer as well as the other $p27^{Kip1}$-deficent carcinomas.

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