Inhibition of MDA-MB-231 Breast Cancer Cell Proliferation and Tumor Growth by Apigenin Through Induction of G2/M Arrest and Histone H3 Acetylation-mediated $p21^{WAF1/CIP1}$ Expression

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ABSTRACT: Apigenin (4′,5,7-trihydroxyflavone), a flavonoid commonly found in fruits and vegetables, has anticancer properties in various malignant cancer cells. However, the molecular basis of the anticancer effect remains to be elucidated. In this study, we investigated the cellular mechanisms underlying the induction of cell cycle arrest by apigenin. Our results showed that apigenin at the nonapoptotic induction concentration inhibited cell proliferation and induced cell cycle arrest at the G2/M phase in the MDA-

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MB-231 breast cancer cell line. Immunoblot analysis indicated that apigenin suppressed the expression of cyclin A, cyclin B, and cyclin-dependent kinase-1 (CDK1), which control the G2-to-M phase transition in the cell cycle. In addition, apigenin upregulated $p21^{WAF1/CIP1}$ and increased the interaction of $p21^{WAF1/CIP1}$ with proliferating cell nuclear antigen (PCNA), which inhibits cell cycle progression. Furthermore, apigenin significantly inhibited histone deacetylase (HDAC) activity and induced histone H3 acetylation. The subsequent chromatin immunoprecipitation (ChIP) assay indicated that apigenin increased acetylation of histone H3 in the $p21^{WAF1/CIP1}$ promoter region, resulting in the increase of $p21^{WAF1/CIP1}$ transcription. In a tumor xenograft model, apigenin effectively delayed tumor growth. In these apigenin-treated tumors, we also observed reductions in the levels of cyclin A and cyclin B and increases in the levels of $p21^{WAF1/CIP1}$ and acetylated histone H3. These findings demonstrate for the first time that apigenin can be used in breast cancer prevention and treatment through epigenetic regulation. Environ Toxicol 00: 000–000, 2016.

Keywords: apigenin; cell cycle arrest; histone H3 acetylation; p21WAF1/CIP1; breast cancer

INTRODUCTION

Cancer is caused by genetic defects such as gene mutations and deletions and chromosomal abnormalities that result in the loss of the function of tumor suppressor genes or gain in the function or hyperactivation of oncongenes. However, increasing evidence indicates that gene expression mediated by epigenetic changes is also crucial in the onset and progression of cancer (Esteller, 2008; Ellis et al., 2009; Sharma et al., 2010). Therefore, targeting epigenetic transcriptional regulation might be a promising strategy for chemoprevention and cancer therapy. Histone acetylation is a valuable mechanism of epigenetic regulation. In normal cells, a balance exists between histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities. Disruption of this balance can lead to the development of cancer. HDAC inhibitors (HDACis) have emerged as valuable molecules that can reverse aberrant epigenetic states associated with cancer. They modulate a wide variety of cellular functions, including cell differentiation, cell cycle progression, apoptosis, and angiogenesis (Bolden et al., 2006; Lin et al., 2006), which can be attributed, in part, to their ability to enhance acetylation of a wide range of proteins, including transcription factors, molecular chaperones, and structural components (Kikuchi et al., 2006; Konstantinopoulos et al., 2007). Currently, increasing evidence indicates that natural products with the ability to inhibit HDAC may play a role in cancer prevention and treatment (Davis and Ross, 2007; Tan and Liu, 2015).

The cell cycle in eukaryotic cells is divided into the G0/G1 phase, S phase, and G2/M phase. Failure of the quality control checkpoints or the loss of balance of the regulatory molecules, including cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CDKIs), play a major role in the development of cancer (McDonald and El-Deiry, 2000). The CDKI $p21^{WAF1/CIP1}$ negatively modulates cell cycle progression by binding to the cyclin–CDK complex and by binding to the proliferating cell nuclear antigen (PCNA) (Cayrol et al., 1998). The dietary component has long been considered to modulate the deregulated cell cycle checkpoint and may contribute to cancer prevention and treatment (Gomathinayagam et al., 2008; Neumann et al., 2014).

Breast cancer is the most common cause of cancer death among women worldwide. Breast cancer is routinely classified by stage, pathology, grade, and expression of estrogen receptor (ER), progesterone receptor (PR), or human epidermal growth factor receptor (Her2/neu). Current successful therapies include hormone-based agents that directly target these receptors (Beaumont and Leadbeater, 2011). Triple-negative breast cancer (TNBC) is a heterogeneous subset of neoplasms that is defined by the absence of these targeted receptors (Elias, 2010). Approximately 15% of globally diagnosed cases of breast cancer are designated as ER, PR, and Her2/neu negative (Hudis and Gianni, 2011). Studies have shown that tumors of this aggressive subtype have a higher histological grade and are more likely to recur earlier at distant sites, resulting in poor overall prognoses (Dent et al., 2007). Thus, new alternative agents are urgently required for TNBC prevention and treatment. The use of naturally occurring compounds for chemoprevention, including those in dietary sources, is a practical approach for breast cancer prevention and treatment (Kim et al., 2009; Ham et al., 2015). Apigenin (4′,5,7-trihydroxyflavone) is a common dietary flavonoid found in fruits, vegetables, and herbs. It has numerous mechanisms of action for cancer prevention and therapy, including estrogenic or antiestrogenic activity, antiinflammation, prevention of oxidation, induction of detoxification enzymes, regulation of the host immune system, and changes in cellular signaling in numerous human cancer cell lines, such as breast, lung, colon, skin, ovarian, thyroid, leukemia, and prostate cancer cell lines (Patel et al., 2007; Shukla and Gupta, 2010). Furthermore, apigenin is an effective proteasome inhibitor in cultured breast cancer cells and breast cancer xenografts (Chen et al., 2007). Our previous study showed that apigenin inhibited hepatocyte growth factor (HGF)-promoted invasion and metastasis of the TNBC cell line MDA-MB-231 by inhibiting the PI3K/Akt pathway and β4 integrin function (Lee et al., 2008). A high concentration of apigenin (>50 μM) has been reported to induce apoptosis in metastatic MDA-MB-231 breast cancer

cells (Chen et al., 2007). However, the molecular basis of the anticancer effect of apigenin remains to be elucidated. In this study, we investigated the cellular mechanisms underlying the action of apigenin in the induction of cell cycle arrest. In the current study, we observed that apigenin induced G2/M arrest in MDA-MB-231 TNBC cells *in vitro* and *in vivo*. In addition, apigenin inhibited HDAC activity and induced histone H3 acetylation to promote $p21^{WAF1/CIP1}$ expression. This suggests that apigenin inhibited breast cancer cell growth through the modulation of epigenetic regulation.

MATERIALS AND METHODS

Chemicals and Reagents

Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Grand Island, NY, USA); antibodies against cyclin D, cyclin E, CDK1, CDK2, CDK4, CDK6, PCNA, and $p21^{WAF1/CIP1}$ (Santa Cruz Biotechnology, Heidelberg, Germany); antibody against β-actin (Sigma Chemical, St. Louis, MO), and antibodies against acetyl H3, acetyl H4, cyclin A, and cyclin B (Cell Signaling Technology, Danvers, MA) were obtained from their respective suppliers. All other chemicals were the highest grade commercially available and supplied either by Merck (Darmstadt, Germany) or Sigma.

MDA-MB-231 Human Breast Cancer Cells

The human TNBC cell line MDA-MB-231 was obtained from the American Type Culture Collection (Manassas, VA). These cells were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, and 1% penicillin–streptomycin–neomycin in a humidified incubator (5% CO_2 air at 37°C). Cultures were harvested and monitored; cells in cell suspensions were counted using a hemocytometer under a phase contrast microscope.

Cell Proliferation and Foci Formation Assays

MDA-MB-231 cells (2×10^5) were plated in 10-cm dishes and incubated for 24 h. Subsequently, the indicated concentrations (0, 10, 20, and 40 μM) of apigenin were added, and the cells were incubated for 24–96 h at 37°C. Thereafter, the cells were stained with 0.4% trypan blue for 5 min at room temperature and were then counted using a hemocytometer under the phase contrast microscope. The number of viable cells was determined using the trypan blue exclusion test. To determine long-term effects, the cells (300 cells per 60-mm dish) were treated with the indicated concentrations (0, 10, 20 and 40 μM) of apigenin for 48 h. Subsequently, the medium was changed twice weekly for 2 weeks. The culture was stained with 5% Giemsa solution. The number of densely stained foci was counted.

Flow Cytometric Analysis

To determine cell cycle distribution, MDA-MB-231 cells (5×10^5) were plated in 10-mm dishes and treated with 40 μM apigenin for 0–36 h. On the other hand, MDA-MB-231 cells (5×10^5) were plated in 10-mm dishes and treated with the indicated concentrations (0, 10, 20, and 40 μM) of apigenin for 48 h. Thereafter, the cells were collected through trypsinization and fixed in ice-cold 70% ethanol at −20°C overnight. The cells were resuspended in phosphate buffered saline (PBS) containing 1% Triton X-100, 0.5 mg mL^{-1} RNase, and 4 μg mL^{-1} propidium iodide (PI) at 37°C for 30 min. The cellular DNA content was determined using flow cytometric analysis of PI-labeled cells. Flow cytometric analysis was performed using an FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with a single argon-ion laser (488 nm). The DNA content of 10,000 cells per analysis was determined using the FACS Calibur flow cytometer. The percentage of cell in the various phases of the cell cycle was determined using ModFit software (Verity Software House, Topsham, ME).

Cell Extract Preparation and Immunoblot Analysis

Cells (1.0×10^6) were seeded into 60-mm dishes in 5 mL of tissue culture medium. After 24-h incubation, cells were treated with the indicated concentrations (0, 10, 20 and 40 μM) of apigenin and then incubated for 0–96 h. Cells were detached, washed once in cold PBS, and suspended in 100 μL lysis buffer (10 mM Tris–HCl, pH 8; 0.32 M sucrose; 1% Triton X-100; 5 mM ethylenediaminetetraacetic acid [EDTA]; 2 mM dithiothreitol [DTT]; and 1 mM phenylmethylsulfonyl flouride). The cell suspension was placed on ice for 20 min and centrifuged at 5000 rpm for 20 min at 4°C. The total protein content was determined using a Bio-Rad protein assay reagent, with bovine serum albumin as the standard; protein extracts were reconstituted in sample buffer (0.062 M Tris–HCl, 2% sodium dodecyl sulfate [SDS], 10% glycerol, and 5% β-mercaptoethanol), and the mixture was boiled for 5 min. Equal amounts (50 μg) of the denatured proteins were loaded into each lane and separated on a 10% or 12% SDS polyacrylamide gel, followed by the transfer of the proteins to nitrocellulose (NC) membranes overnight. The membranes were blocked with 0.1% Tween-20 in Tris-buffered saline containing 5% nonfat dry milk for 20 min at room temperature, and the membranes were incubated with primary antibodies against cyclin D, cyclin E, cyclin B, cyclin A, CDK1, CDK2, CDK4, CDK6, $p21^{WAF1/CIP1}$, PCNA, Ac-H3, and Ac-H4. The membranes were then incubated with a horseradish peroxidase-conjugated goat antirabbit or antimouse antibody. Immunoreactive bands were visualized with enhanced chemiluminescence (ECL), using an ECL commercial kit.

Immunoprecipitation Assay

The effect of apigenin on the interaction of $p21^{waf1/CIP1}$ with PCNA was analyzed using an immunoprecipitation assay. First, MDA-MB-231 cells were treated with 40 μM apigenin for the indicated times (24–96 h) and then lysed with lysis buffer. The lysate protein was immunoprecipitated using monoclonal antibodies against PCNA (2 μg) and protein A/G-agarose (15 μL; Santa Cruz Biotechnology Inc.). Pellet beads were incubated at 4°C overnight. After centrifugation at 12,000 × *g* for 5 min, the immunoprecipitates were washed three times with ice-cold lysis buffer and then subjected to immunoblotting with anti-PCNA and anti-$p21^{WAF1/CIP1}$ antibodies.

HDAC Assay

HDAC activity assays were performed using the colorimetric HDAC assay (BioVision Research Products, Mountain View, CA) according to manufacturer's instructions. Briefly, MDA-MB-231 cells were treated with 0–40 μM of apigenin for 48 h. For HDAC activity, 50 μg of nuclear extracts were diluted in 85 μL of ddH_2O; then, 10 μL of 10× HDAC assay buffer was added, followed by the addition of 5 μL of the colorimetric substrate; samples were incubated at 37°C for 1 h. Subsequently, the reaction was stopped by adding 10 μL of the lysine developer and incubated for an additional 30 min at 37°C. Samples were then read in an enzyme linked immunosorbent assay (ELISA) plate reader at 405 nm.

Nuclear Extract Preparation

MDA-MB-231 cells were lysed by adding 25 μL of 10% NP-40, and the cells were gently passed through a 27-gauge needle. The nuclei were collected through centrifugation at 600 × *g* for 5 min and resuspended in 50 μL of 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.9, containing 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.7 μg mL^{-1} pepstatin, 1 μg mL^{-1} leupeptin, and 10 μg mL^{-1} aprotinin. The tubes were placed on a rotator shaker at 4°C for 30 min, followed by centrifugation at 12,000 × *g* for 5 min at 4°C. The supernatants were used as nuclear extracts and frozen at −80°C until use.

Chromatin Immunoprecipitation Assay

The chromatin immunoprecipitation (ChIP) assay was performed according to the manufacturer's instructions (Upstate Biotechnology, Lake Placid, NY). Briefly, MDA-MB-231 cells were treated with 40 μM apigenin for 0–96 h, and subsequently, the cells were fixed with 37% formaldehyde, sonicated, and immuneprecipitated with antiacetylated histone H-3 antibody. Immunoprecipitated DNA was recovered and used as a template for polymerase chain reaction (PCR). The reaction conditions for PCR were as follows: denaturation at 95°C for 1 min, annealing at 56°C for 1 min, and elongation at 72°C for 1 min, 30 cycles. The PCR products were run on a 2% agarose gel and stained with ethidium bromide. The primer pair used for the $p21^{WAF1/CIP1}$ ChIP assay was as follows: forward, 5′-GGT-GTCTAGGTGCTCCAGGT-3′ and reverse, 5′-GCACTCTCCAGGAGGACACA-3′.

BALB/*c-Nu* Mice

Female athymic nude mice (BALB/*c-nu*) aged 6 weeks were purchased from GlycoNex, Taiwan, and were maintained in cage housing in a specifically designed pathogen-free isolation facility with a 12/12-h light/dark cycle; the mice were provided with rodent chow and water *ad libitum*. All experiments were conducted in accordance with the guidelines of the Animal Ethics Research Board of Chung Shan Medical University.

Animals Experiments

MDA-MB-231 cells (1×10^6 cells at a 1:1 ratio of 100 μL of matrix gel and PBS mixture) were orthotopically injected into the mammary fat pad of female nude mice. After 1 week, the mice were intraperitoneally (i.p.) administered either 100 μL of vehicle (phosphate-buffered saline and DMSO) or 100 μL of vehicle containing apigenin (5 or 25 mg kg^{-1}) every day ($n = 6$) for 8 weeks. The tumor volume was measured every week using a caliper and calculated according to the standard formula: length × $width^2$ × 1/2. Mice were sacrificed at the end of the experiment (9 weeks after cell injection), the tumors were removed, weighed, and photographed. A part of the tumor tissue was immediately frozen, and the rest was fixed in 4% paraformaldehyde, sectioned, and stained with hematoxylin-eosin for analysis under a light microscope. Mitotic cells were identified by microscopic examination and counted in 10 fields (400×) of each tumor sample. All experiments were conducted in accordance with the guidelines and regulations approved by the Institutional Animal Care and Use Committee of Chung-Shan Medical University with a reference number of IACUC Approval No:862.

Statistical Analysis

The experimental data are presented as the mean ± SD. All study data were analyzed using analysis of variance (ANOVA), followed by Dunnett's test. Statistical significance was defined as $P < 0.05$ for all tests.

RESULTS

Apigenin Inhibited MDA-MB-231 Cell Proliferation

It has been reported that high concentrations of apigenin (50–100 μM) induce apoptosis of MDA-MB-231 cells (Chen et al., 2007). Hence, we clarified the antiproliferative

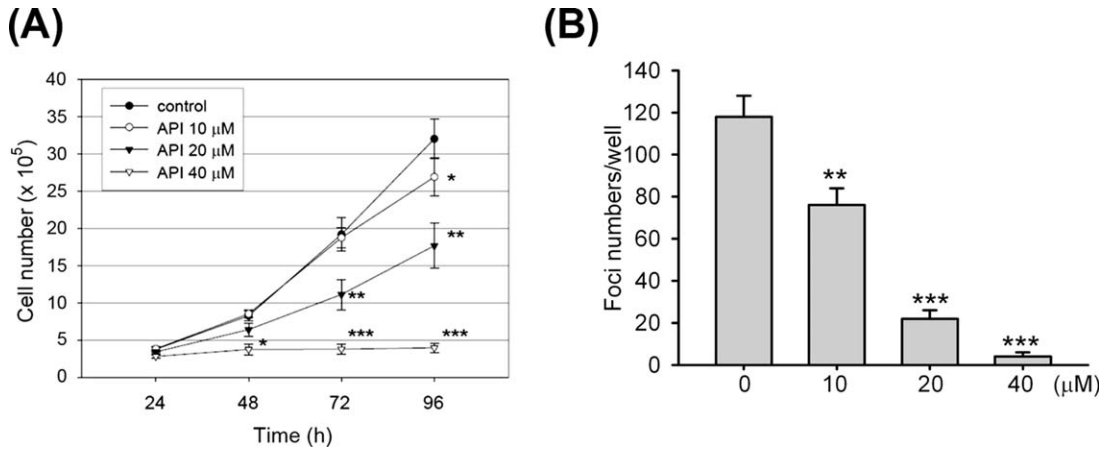

Fig. 1. Effect of apigenin on proliferation of MDA-MB-231 cells. (A) MDA-MB-231 cells (2×10^5) were plated in 10-cm dishes and incubated for 24 h. The indicated concentrations of apigenin were then added, and the cells were incubated for 24–96 h at 37°C. Thereafter, the cells were counted using a hemocytometer. (B) Focus formation assays of MDA-MB-231 cells were performed through treatment with the indicated concentrations of apigenin or 0.2% DMSO (solvent control) for 48 h. The cells were then allowed to grow for 2 weeks to form foci, which were fixed, stained with Giemsa solution, and counted. Data are represented as the mean ± SD of three independent analyses. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, control versus apigenin-treated cells.

potential of apigenin by first treating the cells with apigenin at a lower concentration (40 μM), which we defined as the nonapoptotic induction concentration. Subsequently, we examined the effect of apigenin on cell proliferation by determining the number of viable MDA-MB-231 cells by using the trypan dye exclusion test. As shown in Figure 1(A), 40 μM apigenin significantly inhibited cell growth after treatment for 48 h, and after treatment of the cells for 96 h, apigenin exhibited an antiproliferative effect in a concentration-dependent manner. Furthermore, we examined the effect of apigenin on the long-term growth of MDA-MB-231 cells by using a foci formation assay. This assay showed that the number of foci of cancer cells was reduced in a concentration-dependent manner following treatment with apigenin [Fig. 1(B)].

Apigenin Induced G2/M Arrest in MDA-MB-231 Cells

To determine the phase at which apigenin inhibited cell growth in the cell cycle progression of MDA-MB-231 cells, flow cytometric analysis was performed to determine the DNA content. After fixation and permeabilization, the cells were incubated with PI and assayed for the cell cycle. As shown in Figure 2, apigenin increased the number of cells in the G2/M phase in time-dependent [Fig. 2(B)] and dose-dependent manners [Fig. 2(C)].

Effect of Apigenin on Cell Cycle Regulatory Proteins in MDA-MB-231 Cells

The cell cycle is tightly mediated through a complex network of cell cycle regulatory molecules such as CDKs, cyclins, and CDKI (Meeran and Katiyar, 2008). We examined the levels of cyclins and CDKs in MDA-MB-231 cells by using immunoblot analysis. The results indicated that apigenin did not affect the expression of CDK2, CDK4, CDK6, cyclin D, and cyclin E, which are responsible for the G1 phase transition. However, the levels of cyclin A, cyclin B, and CDK1, which modulate the G2-to-M progression, were reduced in a time-dependent manner in MDA-MB-231 cells [Fig. 3(A)]. Furthermore, we found that the level of the CDKI $p21^{WAF1/CIP1}$ was increased in a time-dependent manner after apigenin (40 μM) treatment [Fig. 3(A)]. Moreover, following treatment with apigenin for the indicated times, the interaction of $p21^{WAF1/CIP1}$ with PCNA increased in MDA-MB-231 cells, as determined by the immunoprecipitation assay [Fig. 3(B)]. These findings implicated that upregulation of $p21^{WAF1/CIP1}$ by apigenin might contribute to the inactivation of PCNA.

Effect of Apigenin on HDAC

Since it has been shown that HDACi strongly activates the expression of $p21^{WAF1/CIP1}$ (Blagosklonny et al., 2002), we evaluated the effect of apigenin on HDAC activity by using ELISA. The result showed that apigenin treatment for 48 h suppressed HDAC activity in MDA-MB-231 cells in a dose-dependent manner [Fig. 4(A)]. Subsequently, we determined which histone acetylation was enhanced after apigenin treatment. As shown in Figure 4(B), H3 acetylation increased in time-dependent [Fig. 4(B)] and dose-dependent [Fig. 4(C)] manners; however, H4 acetylation was not affected by apigenin treatment [Fig. 4(B)]. A previous report indicated that acetylation of H3 in the $p21^{WAF1/CIP1}$ promoter promoted transactivation of $p21^{WAF1/CIP1}$ (Love et al., 2012). To

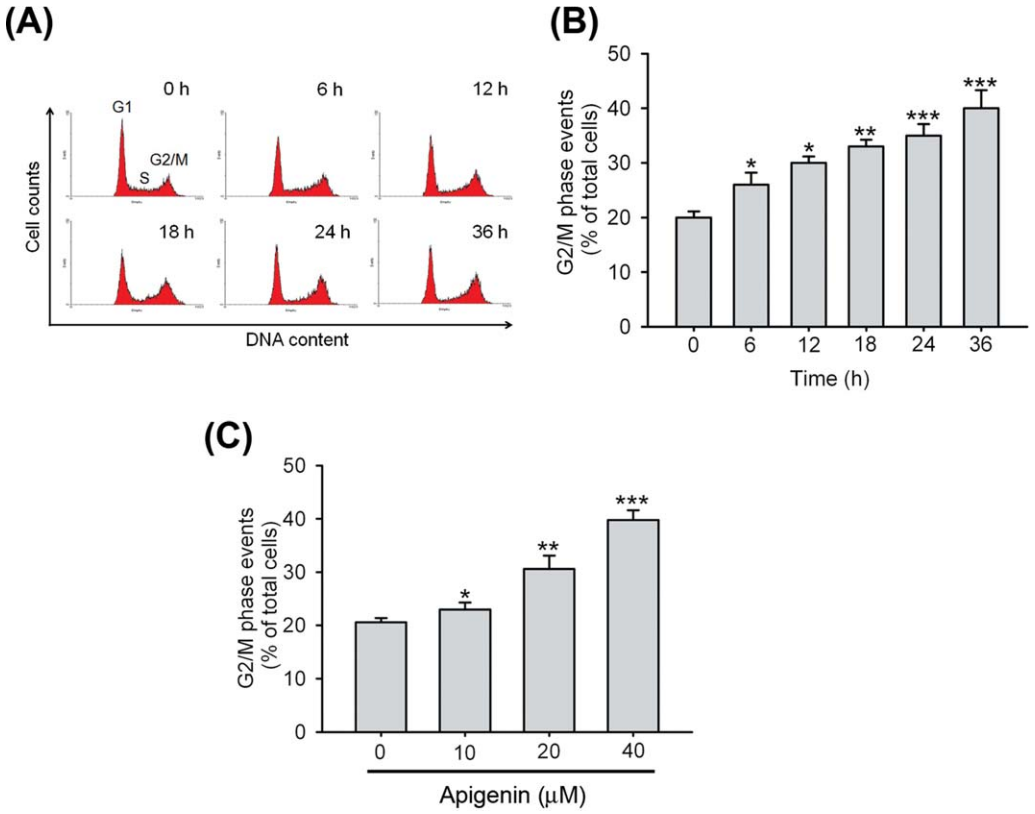

Fig. 2. Effect of apigenin on cell cycle progression in MDA-MB-231 cells. (A) The cells were treated with 40 μM apigenin for the indicated times (0–36 h). Thereafter, the cells were subjected to flow cytometer for cell cycle progression analysis as described in the text. The figure is representative of triplicate experiments. (B) The percentage of cells in the G2/M phase was calculated and expressed as the mean ± SD of three independent experiments. (C) The cells were treated with 0–40 μM apigenin for 48 h. The percentage of G2/M cells was calculated and expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, control versus apigenin-treated cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

evaluate whether histone H3 acetylation promotes the transactivation of $p21^{WAF1/CIP1}$, the ChIP assay was performed in MDA-MB-231 cells treated with apigenin. We also performed immunoblotting with the anti-Ac-H3 antibody, followed by PCR with primers specific for the $p21^{WAF1/CIP1}$ promoter; the results showed that apigenin promoted acetylation of histone H3 in the $p21^{WAF1/CIP1}$ promoter, which might result in its increased transcription [Fig. 4(D)]. These results indicated that apigenin induced upregulation of $p21^{WAF1/CIP1}$ through epigenetic regulation.

Significant Antitumor Growth Effect of Apigenin in an MDA-MB-231 Orthotopic Graft Model

We evaluated the *in vivo* effect of apigenin on tumor growth in nude mice. MDA-MB-231 cells were orthotopically injected into nude mice as described in the Materials and Methods section. All mice appeared healthy, and no body weight loss was noted during apigenin treatment (data not shown). In addition, no signs of toxicity were observed in any of the nude mice, as demonstrated by histological examination of individual organs (data not shown). The growth rates of the MDA-MB-231 orthotopic graft with or without apigenin treatment (treated and control cells, respectively) are shown in Figure 5(A). Evaluation of the tumor volume showed that apigenin significantly and dose dependently inhibited growth. At the end of 9 weeks, the MDA-MB-231 xenografts were excised from each sacrificed animal and weighed. Tumor size and tumor weight in the apigenin-treated mice were inhibited as compared with those in the untreated mice [Fig. 5(B,C)]. These results demonstrate that apigenin exhibits antitumor activity *in vivo*.

Because apigenin treatment significantly reduced the tumor volume and tumor weight, we examined the effect on tumor growth by identifying mitotic cells in tumor sections stained with hematoxylin and eosin. Figure 6(A) shows that apigenin treatment significantly reduced the number of mitotic cells as compared with the number of mitotic cells in the tumors of the untreated mice, indicating that apigenin has an antiproliferative effect. Moreover, we examined the levels of acetylated H3, $p21^{WAF1/CIP1}$, cyclin A, and cyclin

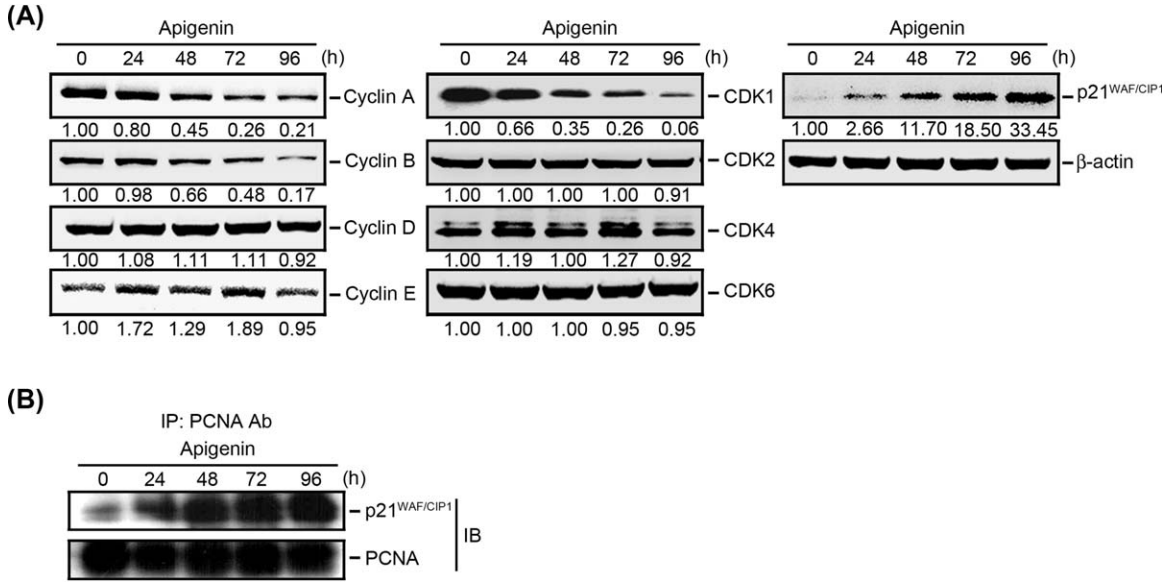

Fig. 3. Effect of apigenin on cell cycle regulatory proteins and the interaction of $p21^{WAF1/CIP1}$ with PCNA in MDA-MD-231 cells. (A) The cells were treated with 40 μM of apigenin for the indicated times. The total protein extracts were prepared for Western blot analysis using the indicated antibodies. (B) The interaction of PCNA with $p21^{WAF1/CIP1}$ was assessed using the immunoprecipitation assay as described in the text.

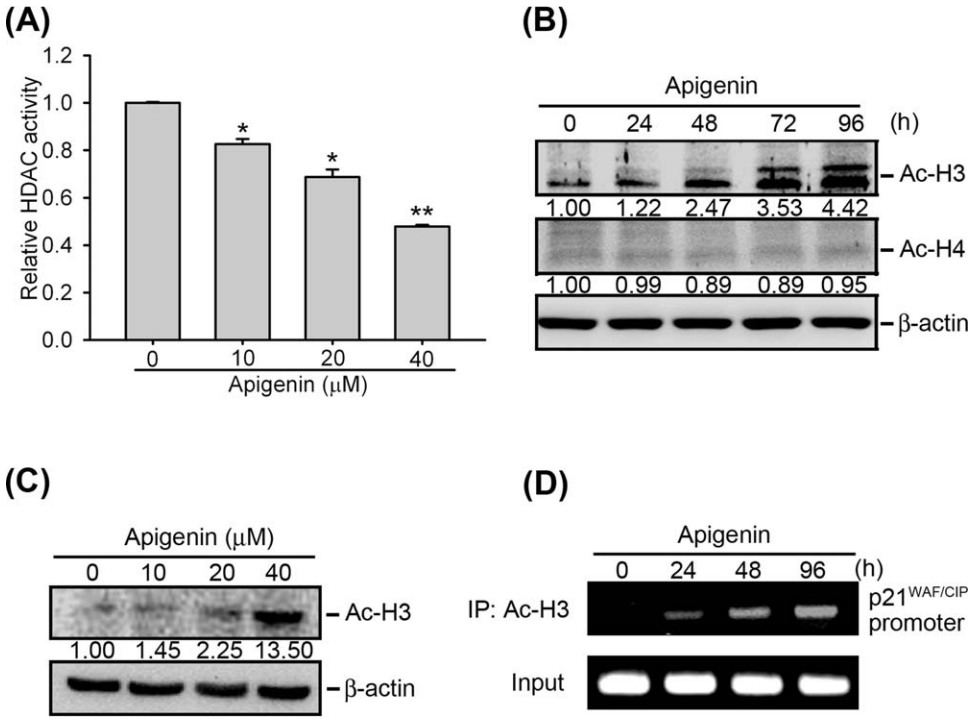

Fig. 4. Effect of apigenin on histone acetylation in MDA-MB-231 cells. (A) After treatment of MDA-MD-231cells with apigenin for 48 h, nuclear extracts were prepared for the HDAC activity assay as described in the text. $^{*}P < 0.01$ and $^{**}P < 0.001$, control versus apigenin-treated cells. (B) MDA-MB-231 cells were treated with 40 μM apigenin for the indicated times (0–96 h). The extracted protein was analyzed using immunoblotting with anti-Ac-H3 and anti-Ac-H4. (C) MDA-MB-231 cells were treated with the indicated concentrations (0–40 μM) of apigenin for 48 h, and the extracted protein was analyzed using immunoblotting with anti-Ac-H3. (D) The ChIP assay with the antibody against acetylated histone H3 and primers for the $p21^{WAF1/CIP1}$ promoter was performed as described in the text. To control input DNA, the $p21^{WAF1/CIP1}$ promoter was amplified from the initial preparations of soluble chromatin without immunoprecipitation.

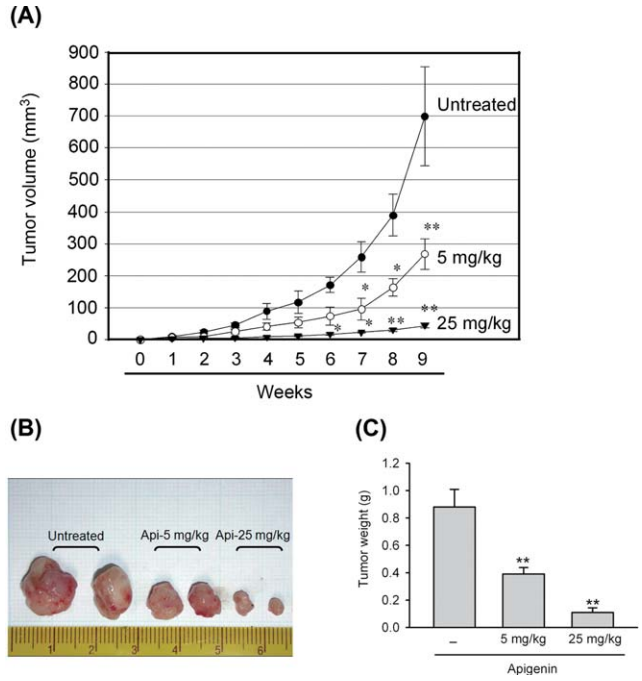

Fig. 5. Inhibitory effects of apigenin on the growth of MDA-MB-231 xenografts of nude mice. After orthotopic implantation of MDA-MB-231 cells for 1 week, the nude mice received vehicle (control) and apigenin (5 and 25 mg kg^{-1}) as described in the Materials and Methods section. (A) The time course effect of apigenin on tumor growth was monitored by measuring the visible tumor sizes at various time points ($n = 6$, $^{*}P < 0.05$, $^{**}P < 0.001$). (B) Mice were sacrificed, and the tumors were removed at the end of the experiment. This figure shows the representative tumor images. (C) The average weight of each tumor was calculated. Column, mean; bar, SD; $n = 6$, for each group ($^{**}P < 0.001$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

B in MDA-MB-231 xenografts harvested from the vehicle- or apigenin-treated mice. Consistent with the results obtained for the cultured MDA-MB-231 cells, apigenin inhibited the growth of MDA-MB-231 xenografts, downregulated cyclin A and cyclin B and upregulated $p21^{WAF/CIP1}$, and increased acetylation of H3 [Fig. 6(B)].

DISCUSSION

Breast cancer is the most common neoplasm in both developed and developing countries (Paik, 2006). It has been suggested that a diet rich in flavonoid-containing plants may contribute to a lower incidence of breast cancer (Middleton et al., 2000; Birt et al., 2001). Apigenin is a flavonoid that possesses remarkable anticancer potential because of its multiple bioactivities such as induction of the detoxification enzyme and cancer cell apoptosis (Birt et al., 1986; Shukla and Gupta, 2010). Chen et al. found that apigenin at a concentration of more than 50 μM can induce apoptosis in cultured MDA-MB-231 cells. In addition, 25 and 50 mg kg^{-1} apigenin can suppress the growth of MDA-MB-231 cells in the xenografts of nude mice, which is associated with proteasome inhibition and apoptosis induction (Chen et al., 2007). In the present study, we used 10–40 μM apigenin in the culture assay and 5 and 25 mg kg^{-1} in the xenografts of the nude mice assay. We found that apigenin at the nonapoptotic induction concentration promoted histone H3 acetylation *in vitro* and *in vivo*, indicating that apigenin exhibits an antiproliferative effect. Histone hyperacetylation is believed to relax the chromatin structure, which allows transcription factors to access promoter sequences. Our results showed that apigenin inhibited HDAC and promoted histone H3 acetylation, leading to the transactivation of $p21^{WAF1/CIP1}$ in MDA-MB-231 cells. $p21^{WAF1/CIP1}$ may inhibit G2-to-M progression by binding with PCNA. This suggests that the chemopreventive role of apigenin involves chromatin remodeling.

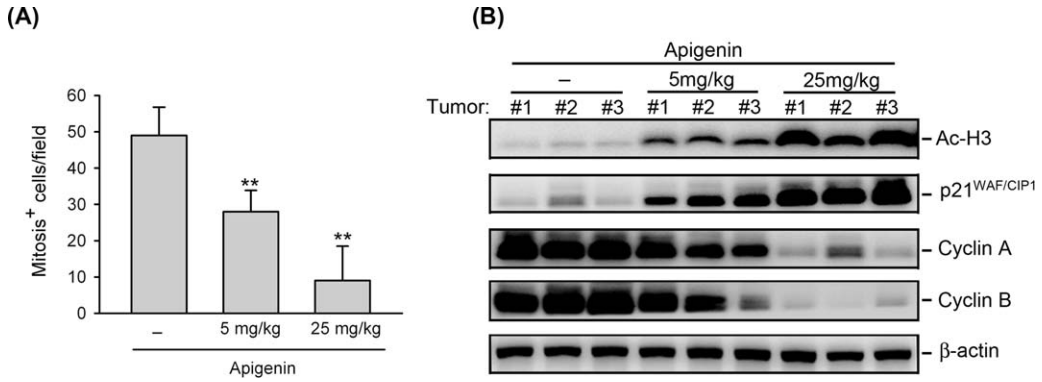

Fig. 6. Inhibitory effects of apigenin on proliferation of MDA-MB-231 cells *in vivo*. (A) Mitotic cells were identified through microscopic examination and counted in 10 fields (×400) of each tumor sample. Column, mean; bar, SD; $n = 6$, for each group ($^{**}P < 0.001$). (B) The tumor was removed, and immunoblot analysis with anti-Ac-H3 and anti-$p21^{WAF1/CIP1}$ and antibodies against cyclin A, cyclin B, and β-actin was performed. The data are presented from three representative mice per group, and each lane contained 50 μg of protein.

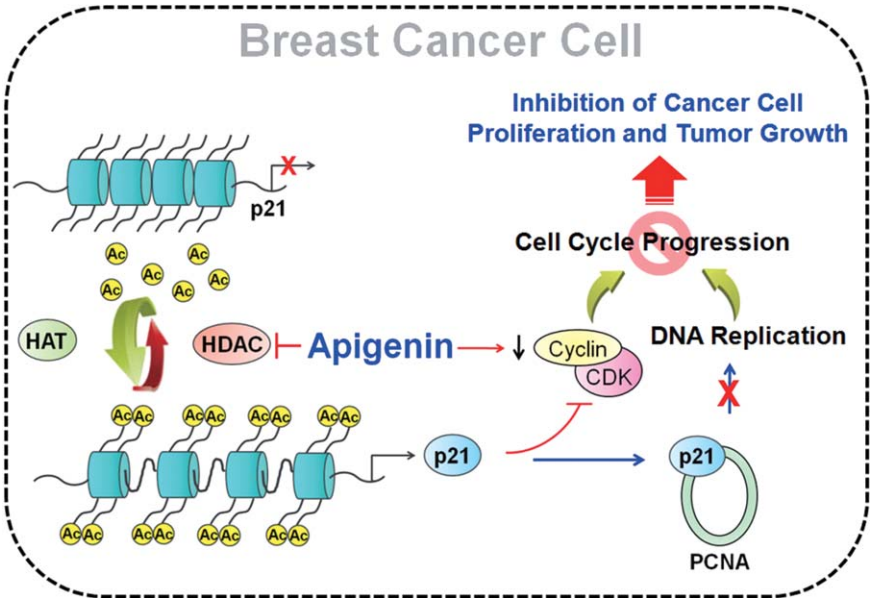

Fig. 7. A working model shows the molecular mechanism underlying the ability of apigenin to suppress the proliferation of MDA-MB-231 breast cancer cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

It is now known that cancer is caused by both genetic alterations (e.g., mutations) and aberrant epigenetic changes (e.g., chromatin remodeling). Epigenetic dysregulation is potentially reversible, making it an attractive target for cancer prevention and treatment. HDACis, such as Trichostatin A, have received considerable attention as anticancer agents because of their ability to induce cell cycle arrest and differentiation and apoptosis of cells in neoplastically transformed cells (Liu et al., 2006). Regarding the therapeutic effect of HDACis on breast cancer, recent reports have shown that in ER-negative breast cancer cells, inhibition of HDAC activity by specific HDACis reactivates the expression of ERα and PR genes, which are aberrantly silenced (Keen et al., 2003; Zhou et al., 2007; Pathiraja et al., 2011). Moreover, Pruitt et al. demonstrated that inhibition of class III HDAC SIRT1 by using the pharmacologic inhibitor splitomicin reactivated epigenetically silenced SFRP1, SFRP2, E-cadherin, and CRBP1 genes in human breast cancer cells (Pruitt et al., 2006). Using the HDAC activity colorimetric assay, we confirmed only that apigenin is a pan-HDACi. The HDAC class that can be specifically targeted by apigenin should be investigated. We will conduct a study investigating whether apigenin modulates genes such as ER and PR genes in addition to the $p21^{WAF1/CIP1}$ gene in TNBC cells. More recently, HDACis have been applied to TNBC treatment. For example, panobinostat, a potent HDACi, has been demonstrated to inhibit G2-to-M progression in several TNBC cell lines *in vitro* and *in vivo* (Tate et al., 2012). Consistent with previous result [36], our current study showed that apigenin had an inhibitory effect on HDAC and induced G2/M arrest in MDA-MB-231 TNBC cells. In addition, apigenin caused G0/G1 arrest in prostate cancer cells through the modulation of the MAPK and PI3K/Akt signaling pathways (Shukla and Gupta, 2007). This suggests that apigenin may inhibit cancer cell cycle progression through the modulation of signaling pathways and epigenetic regulation.

In addition to HDACis alone being used for cancer treatment, recent studies have demonstrated that HDACis play roles in resensitizing cancer cells to agents including those used in chemotherapy (Budman et al., 2011) and radiotherapy (Storch et al., 2010) and autophagy inhibitors (Rao et al., 2012). A recent study indicated that autophagy inhibition can enhance apigenin-induced cell death in breast cancer cells (Rao et al., 2012; Cao et al., 2013). Thus, the combination of apigenin with chemotherapy or radiotherapy for breast cancer treatment is worth investigating in future.

In summary, the present study demonstrated that apigenin possesses anticancer properties in MDA-MB-231 cells *in vitro* and *in vivo*, which are attributed to induction of G2/M arrest through suppressing cyclin A, cyclin B, and CDK1 and inducing histone H3 acetylation-mediated $p21^{WAF1/CIP1}$ expression (Fig. 7). The present study showed for the first time that apigenin is a potential HDACi targeting histone H3. This study revealed that apigenin may be a useful candidate for breast cancer prevention and treatment through epigenetic regulation.

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