Induction of Caspase-dependent Apoptosis by Apigenin by Inhibiting STAT3 Signaling in HER2-overexpressing MDA-MB-453 Breast Cancer Cells

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Abstract. *Background: This study aimed to examine the effect of apigenin on proliferation and apoptosis in HER2-overexpressing MDA-MB-453 breast cancer cells. Materials and Methods: The antiproliferative effects of apigenin were examined by proliferation and MTT assays. The effect of apigenin on apoptotic molecules was determined by western blotting. RT-PCR was performed to measure mRNA levels of HIF-1α and VEGF. ELISA assay was performed to measure intracellular VEGF levels. Immunocytochemistry was performed to evaluate nuclear STAT3 level. Results: Apigenin inhibited the proliferation of MDA-MB-453 cells. Apigenin up-regulated the levels of cleaved caspase-8 and caspase-3, and induced the cleavage of PARP. Apigenin induced extrinsic apoptosis and blocked the activation (phosphorylation) of JAK2 and STAT3. Apigenin inhibited CoCl2-induced VEGF secretion and decreased the nuclear staining of STAT3. Conclusion: Apigenin exerts its antiproliferative activity by inhibiting STAT3 signaling. Apigenin could serve as a useful compound to prevent or treat HER2-overexpressing breast cancer.*

Cancer prevention/treatment through food may be largely feasible by increased consumption of fruits and vegetables. Considerable efforts have been put to identify plant-derived dietary agents which could prevent or treat cancer. A naturally-occurring plant flavone, (4', 5, 7,-trihydroxyflavone), apigenin abundantly presents in common fruits and vegetables including parsley, onions, oranges, tea, chamomile, wheat sprouts and some seasonings (1, 2). Apigenin is known to exert anti-oxidant (2), anti-inflammatory (3), anti-telomerase (4), and anti-depressant activities (5). Notably, apigenin also possesses antitumor properties and, therefore, has special interest for the development of a novel chemopreventive and/or chemotherapeutic agents. It was previously demonstrated that apigenin reduces the tumor volume and mass of androgen-sensitive 22Rv1 and androgen-insensitive PC-3-implanted cells (6). Apigenin also suppresses inducible cyclooxygenase and inducible nitric oxide synthase in mouse macrophages (7) and inhibits ultraviolet light-induced skin carcinogenesis in SKH-1 mice (8). In addition, apigenin induces growth inhibition and apoptosis in a variety of cancer cell lines, including breast (9), lung (10), colon (11, 12), prostate (13), leukemia (14), and pancreatic (15) cells.

Apoptosis plays an important role in determining cellular fate; it participates in development, cellular homeostasis, and both physiological as well as pathological processes (16-18). Caspases are aspartate-specific cysteine proteases and members of the interleukin-1β-converting enzyme family and essential in cells for apoptosis (16, 17). The caspase cascade system plays vital roles in the induction, transduction and amplification of intracellular apoptotic signals (16, 17). The apoptotic-signaling cascade is divided into two major pathways, extrinsic and intrinsic. The extrinsic pathway is related to the activation of death receptors, such as Fas and the tumor necrosis factor receptors (TNFRs) and the cleavage of caspase-8 and caspase-3 (19-21). The intrinsic pathway is related to changes in the mitochondrial membrane potential ($\Delta_{\psi m}$), the mitochondrial permeability transition, and the cleavage of caspase-9 and caspase-3 (22). In both the extrinsic and intrinsic pathways, caspase-3 is responsible for the cleavage of poly (ADP-ribose) polymerase (PARP) during cell death (23).

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Human epidermal growth factor receptor-2 (HER2)-positive breast cancers tend to grow more quickly than HER2-negative breast cancers. In HER2-positive breast cancers, hormonal receptors expression is associated with a poor prognosis despite the hormone therapy (24). HER2 amplification or overexpression is a risk factor for recurrence (24). Between 15 and 25 out of every 100 women with breast cancer (15-25%) have HER2-positive cancers (25). Effective treatments called targeted (or sometimes biological) therapies have been developed to treat HER2-positive breast cancer. The drug most commonly used is trastuzumab (Herceptin®) (26). Trastuzumab induces the down-regulation of HER-2/Neu, leading to the disruption of receptor dimerization and signaling (27). Trastuzumab also causes arrests during G_1 phase of the cell cycle and inhibits the phosphorylation of p27Kip1, resulting in the suppression of cdk2 activity and reduced proliferation (28). Trastuzumab suppresses angiogenesis by both the induction of anti-angiogenic factors and the repression of pro-angiogenic factors. However, many women do not respond to trastuzumab or develop resistance to this drug (29). This has resulted in significant efforts to identify other compounds that can effectively treat HER-2-overexpressing breast cancer.

Previously, we reported that genistein and quercetin induce apoptosis *via* the extrinsic pathway inhibiting nuclear factor-kappa B (NF-κB) signaling in HER2-transfected MCF-7 cells (30). We also reported that apigenin promotes apoptosis *via* the extrinsic pathway, inducing p53 and inhibiting signal transducer and activator of transcription-3 (STAT3) and NF-κB signaling in HER-2-transfected MCF-7 cells (31). In the present study, we investigated whether apigenin produces apoptosis-inducing cleavage of caspases and PARP and regulating STAT3 signaling. We also investigated whether apigenin regulates the levels of VEGF which is a STAT3 target gene. Our study will provide new insight on the role of phytoestrogens in the prevention or treatment of HER2-overxpressing breast cancer.

Materials and Methods

Compounds. Apigenin (4',5,7-trihydroxyflavone) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Apigenin was dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in the controls and each sample did not exceed 0.1%. We found that 0.1% DMSO did not affect the cell growth rate compared to 0% DMSO (no treatment) in the breast cancer cells (data not shown). The caspase-8 inhibitor Z-IETD-*fmk* and the caspase-9 inhibitor Z-LEHD-*fmk* were obtained from R&D Systems, Inc. (Minneapolis, MN, USA). The STAT3 inhibitor S31-201 was obtained from Calbiochem (Billerica, MA, USA). An EZ-western chemiluminescent detection kit was purchased from Daeillab Service Co. (Seoul, Korea).

Cell culture. The human breast cancer cell line MDA-MB-453 (American Type Culture Collection, Manassas, VA, USA) was cultured in RPMI containing 50 U/ml penicillin, 50 mg/ml streptomycin and 10% FBS (Welgene, Daegu, Korea) at 37˚C in an atmosphere of 5% CO_2.

Antibodies. Primary antibodies against FAS, cleaved caspase-8, caspase-3, cleaved caspase-3, PARP, and HER2 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Primary antibodies against Bcl-2, BAX, p53, phospho-p53 (Ser15), p21 and VEGF were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Primary antibodies against Bcl-xL and HIF-1α was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Primary antibodies against STAT3, phospho-STAT3 (Tyr705), phospho-JAK2 (Tyr1007/Tyr1008) were obtained from Upstate-Millipore (Billerica, MA, USA). The anti-tubulin antibody was from Sigma Chemical Co. Horseradish peroxidase (HRP)-conjugated secondary antibodies (mouse and rabbit) were purchased from Calbiochem (San Diego, CA, USA).

Cell proliferation assay. Cells were seeded in 12-well culture plates at a density of 8×10^4 cells/well. After the cells were exposed to different concentrations of apigenin (0-60 μM) and incubated for 3 days, they were harvested by trypsinization, re-suspended in 1-2 ml medium, and counted using a hemocytometer.

MTT assay. Cells were seeded in 96-multiwell culture plates at densities of 5×10^3 - 7×10^3 cells/well and incubated overnight at 37˚C. Then, they were treated with different concentrations of apigenin (0-60 μM) for 24 h, 48 h, or 72 h. After incubation, MTT reagent (0.5 mg/ml) was added to each well, and the plates were incubated in the dark at 37˚C for 2 h. At the end of the incubation, the medium was removed, the resulting formazan was dissolved in DMSO, and the optical density was measured at 570 nm using an ELISA plate reader.

Cell-cycle analysis by flow cytometry. Cells were harvested with 0.25% trypsin and washed once with phosphate buffered saline (PBS). After centrifugation, the cells were fixed in cold 95% ethanol with 0.5% Tween-20, and stored at –20˚C for at least 30 min. The cells were incubated in 50 μg/ml propidium iodide (PI) (including 1% sodium citrate and 50 μg/ml RNase A) at room temperature in the dark for 30 min. Analysis of the apoptotic cells was performed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA), and the data were analyzed using CellQuest software.

Immunocytochemistry. Cells (6×10^4 cells/well) were seeded in 8-well chamber slides, incubated for 24 h at 37˚C and treated with apigenin (60 μM) in the presence or absence of $CoCl_2$ for another 24 h. The cells were fixed with 4% paraformaldehyde for 30 min and treated with 3% hydrogen peroxide (H_2O_2) in methanol for 20 min to quench the endogenous peroxidase activity. The cells were washed with PBS, blocked with 5% BSA in PBS for 1 h and incubated with the anti-STAT3 primary antibody (1:100 dilution) overnight at 4˚C. After washing with PBS, the cells were incubated with the anti-rabbit biotin-conjugated secondary antibody for 1 h at room temperature. Then, the cells were treated with Vectastain ABC reagent (Vector Laboratories, Inc. Burlingame, CA, USA) for 30 min at 4˚C and stained with diaminobenzidine tetrachloride (DAB) and hematoxylin. The cells were mounted with mounting medium and subsequently analyzed by microscopy.

Measurement of VEGF secreted from MDA-MB-453 cells by ELISA. To assess the level of VEGF in the MDA-MB-453 cell supernatants, the cells were treated with apigenin (0-60 μM) in the presence or absence of $CoCl_2$ (100 μM) to mimic hypoxia. After 24 h, the media were collected, centrifuged to remove the cellular debris, and stored

at –70˚C until assayed for VEGF. The amount of VEGF secreted into the culture medium was measured by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Briefly, 96 well plates were coated with capture antibody in ELISA coating buffer and incubated overnight at 4˚C. The plates were then washed with PBS with 0.05% Tween 20 (PBS-T) and subsequently blocked with 10% FBS in PBS for 1 h at 20˚C. Serial dilutions of standard antigen or sample in dilution buffer (10% FBS in PBS) were added to the plates, and the plates were incubated for 2 h at 20˚C. After washing, biotin-conjugated anti-mouse IgE and streptavidin-conjugated horseradish peroxidase (SAv-HRP) were added to the plates, and the plates were incubated for 1 h at 20˚C. Finally, the tetramethylbenzidine (TMB) substrate was added to the plates, and after 15 min of incubation in the dark, 2 N H_2SO_4 was added to stop the reaction. The optical density was measured at 450 nm on an automated ELISA reader.

Western blot analysis. Cells were lysed in modified RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM NaF, 1 mM Na_3VO_4, and a protease inhibitor mixture). The lysates were cleared by centrifugation at 13,000 rpm for 15 min, and the supernatants were collected. The protein concentration was quantified using a Bradford Protein Assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein lysates were used for western blot analysis with the indicated antibodies. The immunoreactive protein bands were detected using an EZ-Western Detection kit (Daeillab Service Co., Seoul, Korea).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Whole-cell lysates under diverse conditions were prepared by washing with ice-cold PBS. Total RNA was isolated using Trizol reagent (iNtRON biotechnology, Seong-Nam, Korea). Total RNA was treated with 2 units of RNase-free DNase at 37˚C for 30 min, extracted with phenol/chloroform/isoprophanol, and precipitated with ethanol. The RNA concentration was determined by measuring the absorbance at 260 nm using a nanodrop, and the ratio of absorbance at 260 nm and 280 nm was 1.8 or higher. cDNA was synthesized from 2 μg of total RNA as a template in 20 μL reaction mixture containing 5X first strand buffer, 0.1 M DTT, 10 mM dNTP, and 200 unit M-MLV reverse transcriptase (iNtRON biotechnology). cDNA was incubated at 42˚C for 1 h and inactivated at 95˚C for 5 min. After inactivation, the cDNA was stored at –20˚C until use. RT-PCR was performed by co-amplification of the genes with a reference gene by use of the cDNA template and corresponding gene-specific primer sets. The primer sequences are shown in Table I. PCR was conducted out in a total volume of 25 μL containing 5 μL of cDNA solution, 25 μM of sense primers, and 25 μM of antisense primers, 1×PCR buffer, 2.5 mM $MgCl_2$, and 2.5 unit Taq DNA polymerase (Takara Korea, Seoul, Korea). The sequencing involved 30 cycle at 94˚C, 45 sec for denaturation, 58˚C, 45 sec for annealing, and 72˚C, 45 sec for extension. The resulting PCR products were resolved on 1% agarose gels containing ethidium bromide.

Statistical analysis. All experiments were performed in triplicate. The data for the cell proliferation assay, MTT assay and ELISA assay are expressed as the mean±standard deviation (SD). The standard deviations for all of the measured biological parameters are displayed in the appropriate Figures. A student's *t*-test was used for single variable comparisons, and a *p*-value of <0.05 was considered statistically significant.

Table I. *The sequence of primers used*.

Primers	Sequence
HIF-1α	Forward 5' TCA CCA CAG GAC AGT ACA GGA TGC 3'
	Reverse 5' CCA GCA AAG TTA AAG CAT CAG GTT CC 3'
VEGF	Forward 5' AAG GCC CAC AGG GAT TTT CT 3'
	Reverse 5' AGG AGG GCA GAA TCA TCA CG 3'
GAPDH	Forward 5' CGG CCA TCA CGC CAC AGT TT 3'
	Reverse 5' CGT CTT CAC CAC CAT GGA GA 3'

RT-PCR was performed by coamplification of the genes with a reference gene by use of the cDNA template and corresponding gene-specific primer sets.

Results

Apigenin suppresses the growth of MDA-MB-453 cells. We investigated the growth suppressive activity of three different phytoestrogens (apigenin, quercetin and genistein) in MDA-MB-453 cells using a cell proliferation assay. As shown in Figure 1A, apigenin, genistein and quercetin significantly inhibited MDA-MB-453 cell proliferation in a dose-dependent manner (0-100 μM) after 72 h of treatment. In addition, the time-dependent growth suppressive activity of apigenin was measured by the MTT assay, as shown in Figure 1B. It seems that the proliferation assay was more sensitive than the MTT assay with respect to measuring the intensity of the cell growth inhibition, as shown in Figure 1A and 1B. Moreover, the growth inhibition induced by apigenin was verified by microscopic observation. The results in Figure 1C show that apigenin effectively inhibited the growth rate of MDA-MB-453 monolayer cells after 72 h of treatment. Of note, apigenin also induced morphological changes in these cells (Figure 1C).

The growth-suppressive activity of apigenin is accompanied by an increase in the subG_0/G_1 apoptotic population in MDA-MB-453 cells. To investigate whether apigenin inhibits cell proliferation by promoting changes in cell-cycle progression, the effect of apigenin on the cell-cycle profile was assessed in MDA-MB-453 cells. For this purpose, cells were treated with apigenin (0-60 μM) for 72 h and then analyzed for cell-cycle stage by flow cytometry. The results demonstrated that apigenin induced an increase in the sub G_0/G_1 apoptotic population in MDA-MB-453 cells (Figure 2).

Apigenin induces apoptosis via a caspase-dependent pathway in MDA-MB-453 cells. In this step, we investigated whether apigenin activates caspase-dependent apoptosis by measuring the expression of caspase-8, caspase-3, and PARP. We observed that apigenin up-regulated the levels of cleaved caspase-8 and caspase-3, and induced the cleavage of PARP in SKBR3 cells (Figure 3A). We also found that the cleavage

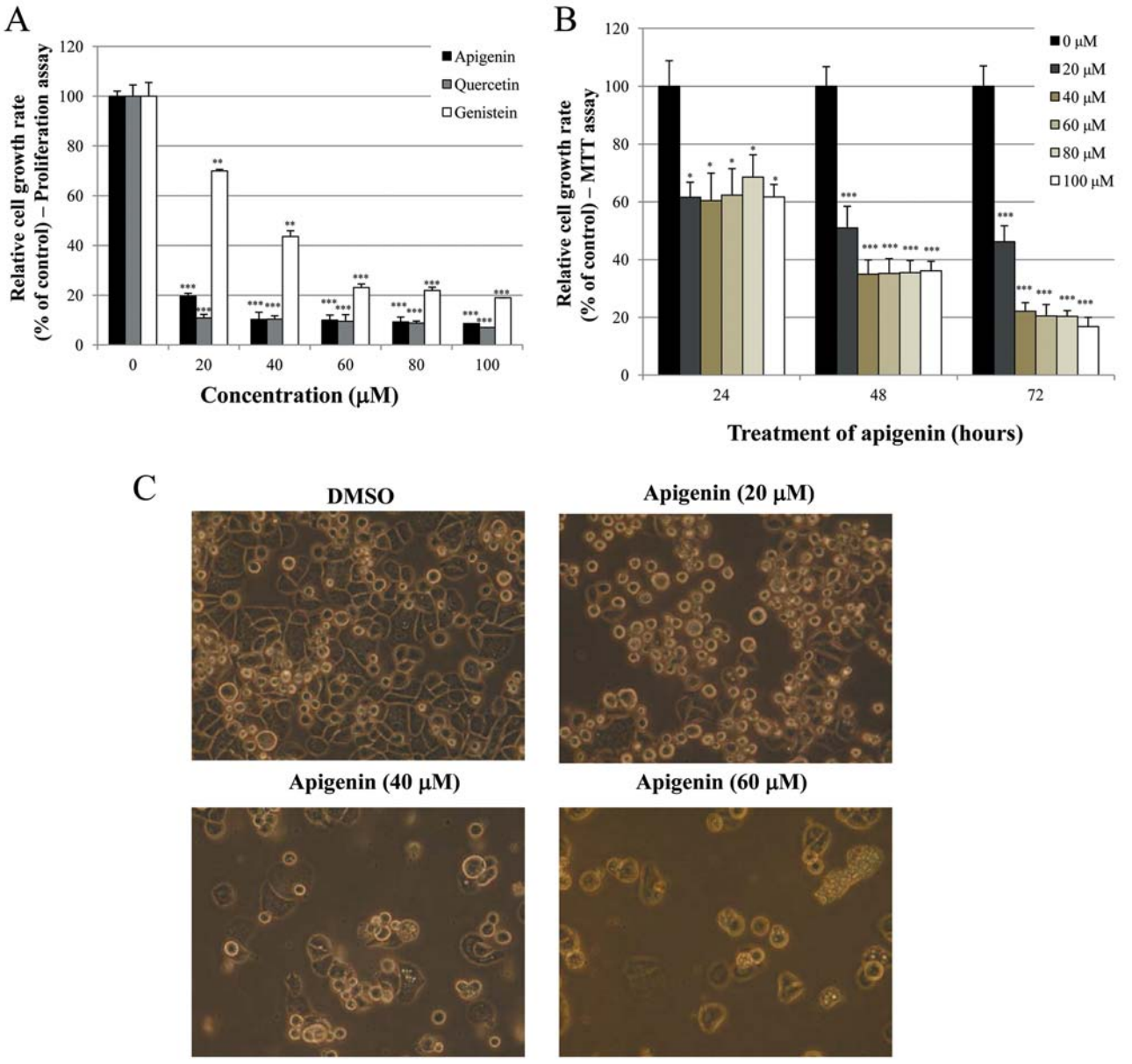

Figure 1. *Effect of apigenin on MDA-MB-453 cell growth. (A) MDA-MB-453 cells were treated with different doses of apigenin, genistein and quercetin (0-100 μM). After 72 h, cell viability was assessed using a cell proliferation assay. (B) MDA-MB-453 cells were treated with different doses of apigenin (0-100 μM). The relative cell growth rate was measured by the MTT assay after 24 h, 48 h and 72 h. The growth rate of the vehicle-treated cells was set to 100%, and the relative decrease in cell viability resulting from the phytoestrogen treatment was expressed as a percentage of the control. (C) MDA-MB-453 cells were treated with different doses of apigenin (0-60 μM) for 72 h and photographed by phase-contrast microscopy (original magnification, ×40). Control cells were treated with DMSO-alone. Data are shown as the means of three independent experiments (error bars denote SD). *p<0.05, **p<0.01, ***p<0.001.*

of caspase-8, caspase-3, and PARP was inhibited by the caspase-8 inhibitor Z-IETD-*fmk* and the caspase-9 inhibitor Z-LEHD-*fmk* (Figure 3B), but apigenin prevented this inhibition and was able to induce the cleavage of caspase-3 and PARP in the presence of Z-IETD-*fmk* and Z-LEHD-*fmk* (Figure 3B). Moreover, the caspase-8 and caspase-9 inhibitors did not suppress cell growth, but apigenin was able to induce apoptosis even in their presence (Figure 3C). These results confirm that apigenin strongly promotes apoptosis *via* a caspase-dependent mechanism.

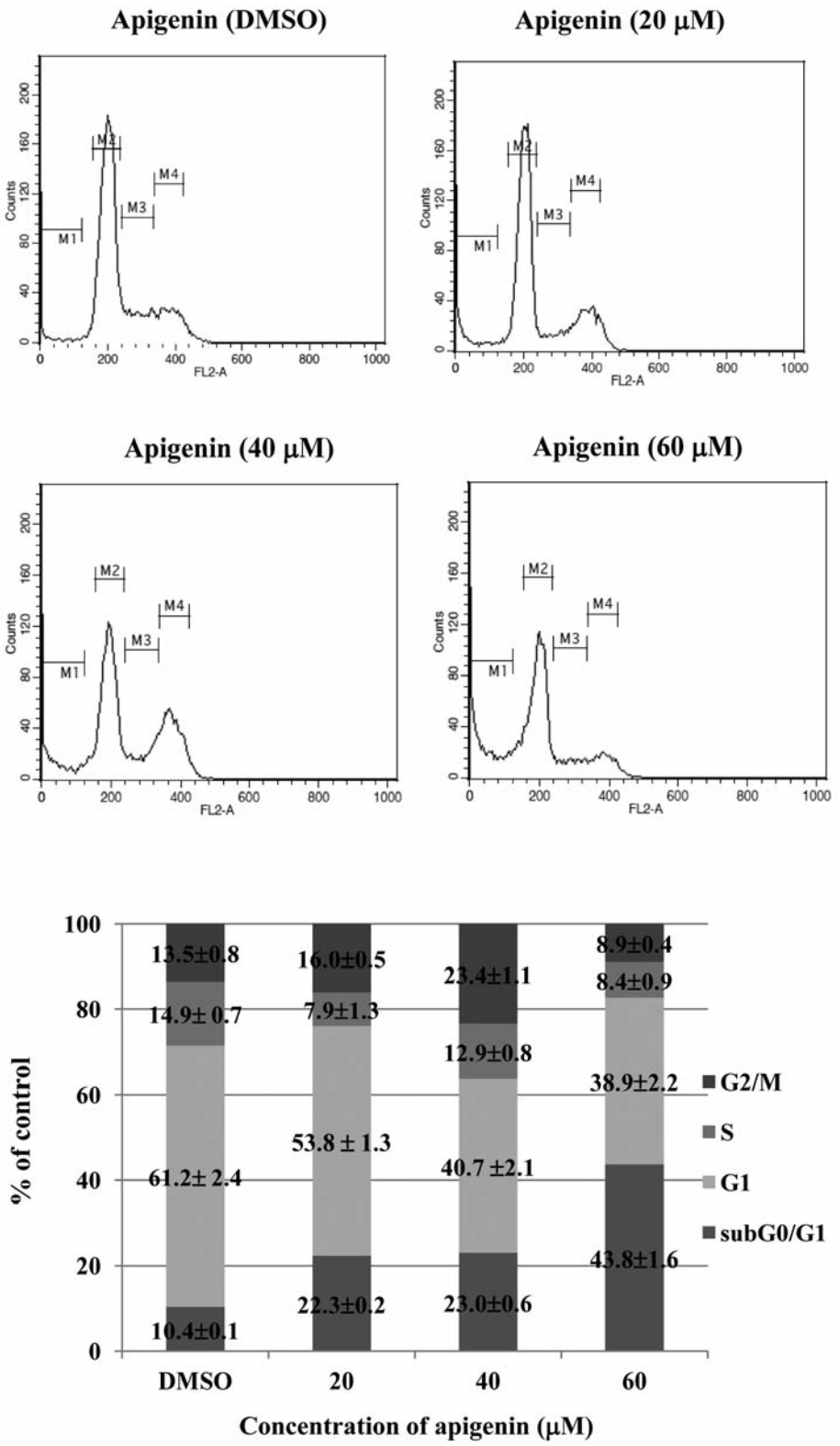

Figure 2. *Effect of apigenin on the cell cycle and subG$_0$/G$_1$ apoptotic population of MDA-MB-453 cells. (A) MDA-MB-453 cells were treated with apigenin (0-60 μM) and fixed for 72 h for flow cytometry. Propidium iodide-labeled nuclei were analyzed for DNA content. (B) The subG$_0$/G$_1$ apoptotic population and the G$_1$, S and G$_2$/M phase populations were quantified using DNA histograms. The data shown are representative of three independent experiments that gave similar results.*

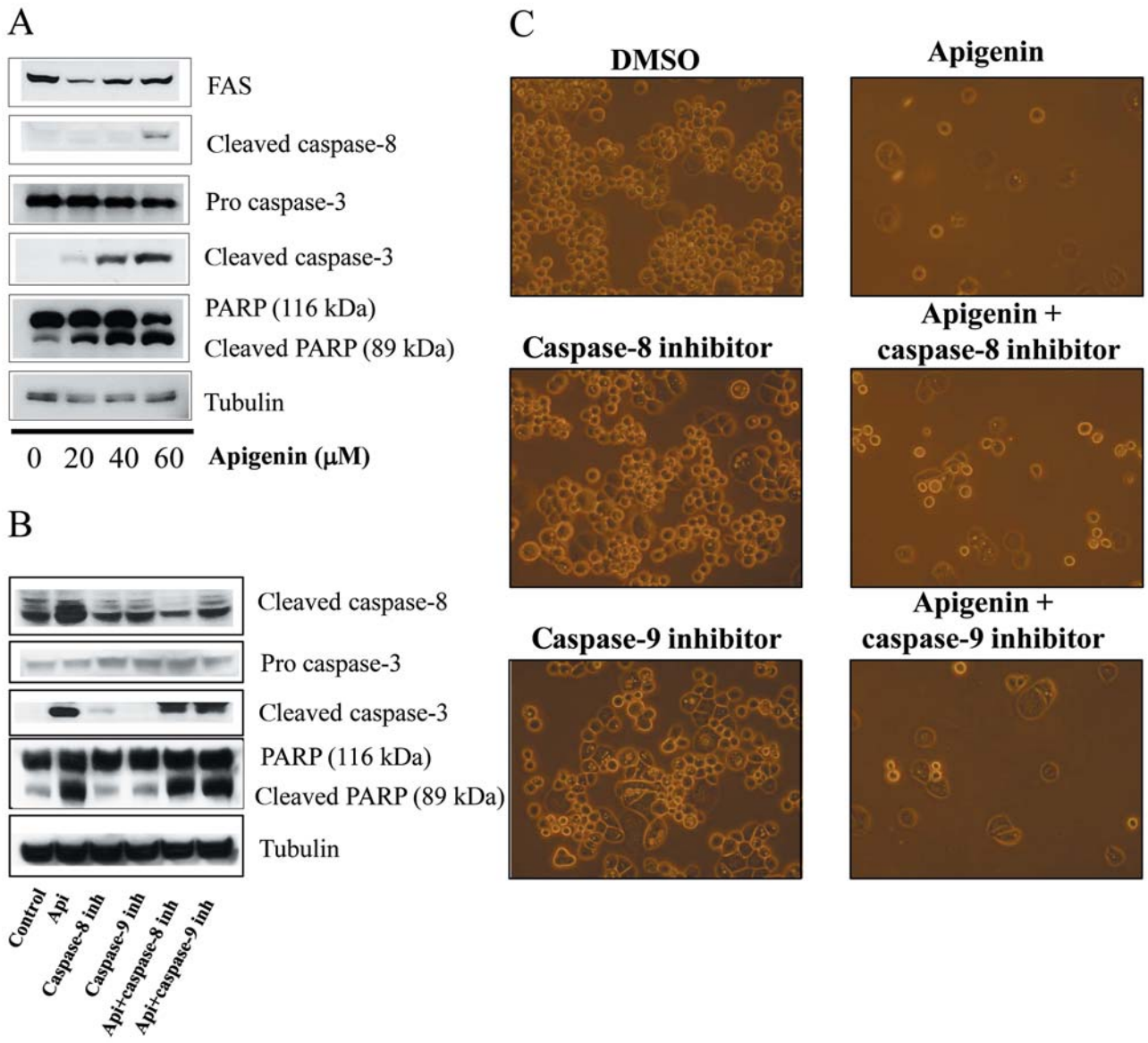

Figure 3. *Effect of apigenin on the expression of apoptotic molecules in MDA-MB-453 cells. (A) Apigenin induces apoptosis via caspase-dependent apoptosis pathway in MDA-MB-453 cells. MDA-MB-453 cells were treated with apigenin (0-60 μM) for 24 h. Whole-cell lysates were analyzed by western blotting with anti-FAS, anti-cleaved caspase-8, anti-caspase-3, anti-cleaved caspase-3, anti-PARP and anti-tubulin antibodies. The data shown are representative of three independent experiments that gave similar results. (B) Effect of caspase-8 and caspase-9 inhibitors on apigenin-induced apoptosis in MDA-MB-453 cells. MDA-MB-453 cells were exposed to 60 μM apigenin with or without the caspase-8 inhibitor (40 μM) or the caspase-9 inhibitor (40 μM) for 24 h, the cell lysates were separated by SDS-PAGE, and western blotting with specific antibodies was performed (anti-cleaved caspase-8, anti-caspase-3, anti-cleaved caspase-3, anti-cleaved PARP, and anti-tubulin). The data shown are representative of three independent experiments that gave similar results. (C) Effect of caspase-8 and caspase-9 inhibitors on MDA-MB-453 cell proliferation. MDA-MB-453 cells were exposed to 60 μM apigenin with or without the caspase-8 inhibitor (40 μM) or the caspase-9 inhibitor (40 μM) for 72 h and photographed by phase contrast microscopy (original magnification, ×40).*

Apigenin induces extrinsic apoptosis in MDA-MB-453 cells. Next, we investigated whether apoptosis induced by apigenin occurs *via* the extrinsic apoptosis pathway in MDA-MB-453 cells. For this purpose, we measured the levels of BCL2 family members (BAX, Bcl-2, and Bcl-xL) which are important in non-extrinsic (intrinsic mitochondrial) apoptosis pathway. We found that apigenin failed to regulate the levels of BAX, Bcl-2, and Bcl-XL in MDA-MB-453 cells as seen in Figure 4A and 4B. We also measured the loss of mitochondrial transmembrane

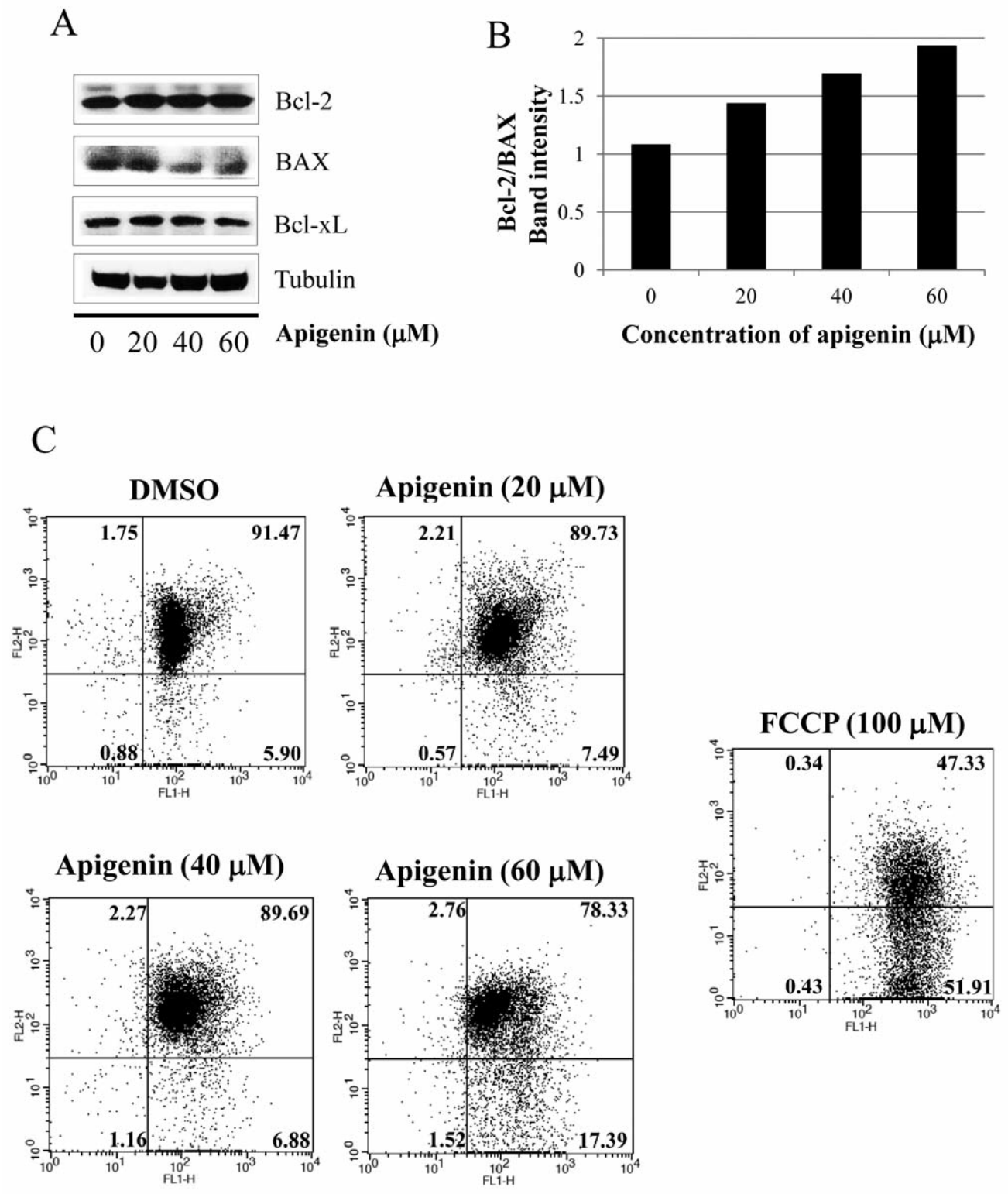

Figure 4. *Apigenin induces apoptosis via the extrinsic pathway in MDA-MB-453 cells. (A) and (B) analysis of intrinsic apoptosis-related molecules. MDA-MB-453 cells were treated with apigenin (0-60 μM) for 24 h. Total proteins were analyzed by western blotting with anti-Bcl-2, anti-BAX, anti-Bcl-×L and anti-tubulin antibodies. (C) MDA-MB-453 cells were incubated with apigenin (0-60 μM) for 72 h and were dyed with JC-1 (4 μg/ml). The data were analyzed by FACSCalibur flow cytometry measuring the green fluorescence and red fluorescence at 514/529 nm (FL-1) and 585/590 nm (FL-2), respectively. The data shown are representative of three independent experiments that gave similar results.*

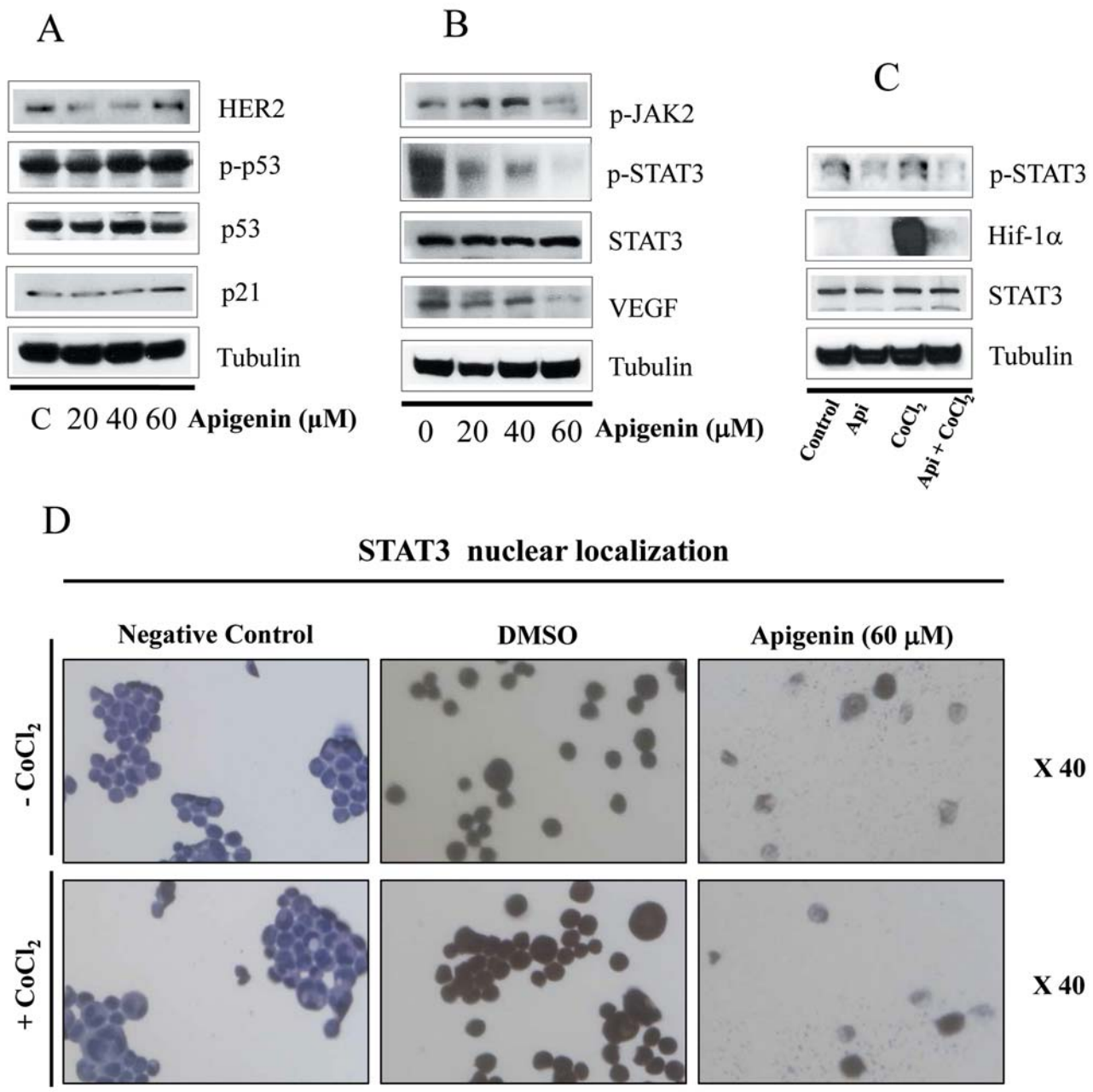

Figure 5. *Effect of apigenin on STAT3 activation in MDA-MB-453 cells. (A) MDA-MB-453 cells were treated with apigenin (0-60 μM) for 24 h. Whole-cell lysates were analyzed by western blotting with anti-HER2, anti-p-p53, anti-p53, anti-p21 and anti-tubulin antibodies. (B) MDA-MB-453 cells were treated with apigenin (0-60 μM) for 24 h. Whole-cell lysates were analyzed by western blotting with anti-p-JAK2, anti-p-STAT3, anti-STAT3, anti-VEGF, and anti-tubulin antibodies. (C) MDA-MB-453 cells were treated with apigenin (60 μM) for 24h in the presence or absence of $CoCl_2$ (4 h). Whole-cell lysates were analyzed by western blotting with anti-phospho-STAT3, anti-HIF-1α, anti-STAT3, and anti-tubulin antibodies. (D) MDA-MB-453 cells were treated with apigenin (60 μM) for 24 h in the presence or absence of $CoCl_2$ and then submitted to immunocytochemistry for detection of nuclear STAT3. The data shown are representative of three independent experiments that gave similar results.*

potential ($\Delta\Psi_m$) using JC-1. JC-1 is able to selectively enter mitochondria and reversibly-transforms color from red to green when the membrane potential decreases. In non-apoptotic cells with high mitochondrial $\Delta\Psi_m$, JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. On the other hand, in apoptotic cells (especially mitochondria-mediated apoptotic cells) with low $\Delta\Psi_m$, JC-1 remains in the monomeric form, which

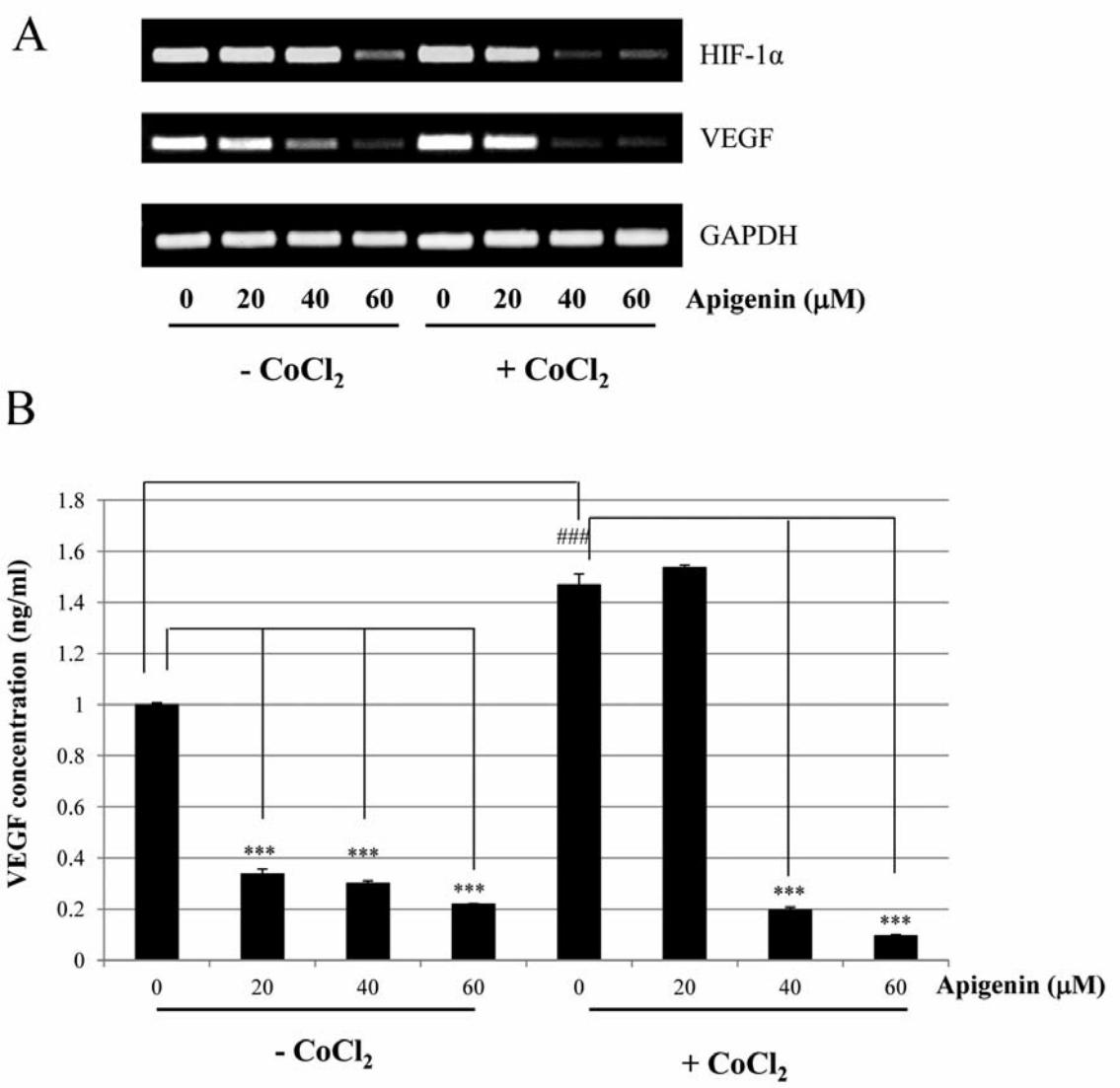

Figure 6. *Effect of apigenin on the levels of HIF-1α and VEGF in MDA-MB-453 cells. (A) MDA-MB-453 cells were treated with apigenin (0-60 μM) for 24 h in the presence or absence of $CoCl_2$, and the mRNA levels of HIF-1α and VEGF were measured by RT-PCR. (B) MDA-MB-453 cells were treated with apigenin (0-60 μM) for 24 h in the presence or absence of $CoCl_2$, and the intracellular VEGF concentration was measured by ELISA. The data shown are representative of three independent experiments that gave similar results.*

shows only green fluorescence. In our study, apigenin did not induce a low mitochondrial transmembrane potential ($\Delta_{\Psi m}$), showing relatively weak green fluorescence (DMSO; 5.90%, Api 20 μM; 7.49%, Api 40 μM; 6.88%, Api 60 μM; 17.39%) as compared to FCCP (positive control: 51.91%) (Figure 4C). These results demonstrate that apigenin does not induce apoptosis *via* the intrinsic mitochondrial pathway but induces apoptosis *via* the extrinsic pathway in MDA-MB-453 cells.

Effect of apigenin on STAT3 activation in MDA-MB-453 cells. Figure 5A shows that MDA-MB-453 cells express HER2 and apigenin slightly up-regulates phospho-p53 (p-p53) and p21 (p53 target gene). In Figure 5(B), we investigated whether apigenin affects STAT3 signaling by measuring levels of p-STAT3 and VEGF (*STAT-3* target gene). We found that apigenin reduced the expression of p-STAT3 as well as p-JAK2 (an upstream kinase of STAT3) (Figure 5B). Apigenin also reduced the level of VEGF (Figure 5B). Since STAT3 is a potential modulator of HIF-1α, we observed the relationship between STAT3 and HIF-1α. We found that apigenin suppressed the expression of p-STAT3 and Hif-1α that was up-regulated by $CoCl_2$ (hypoxia mimic) (Figure 5C). Immunocytochemical staining indicated

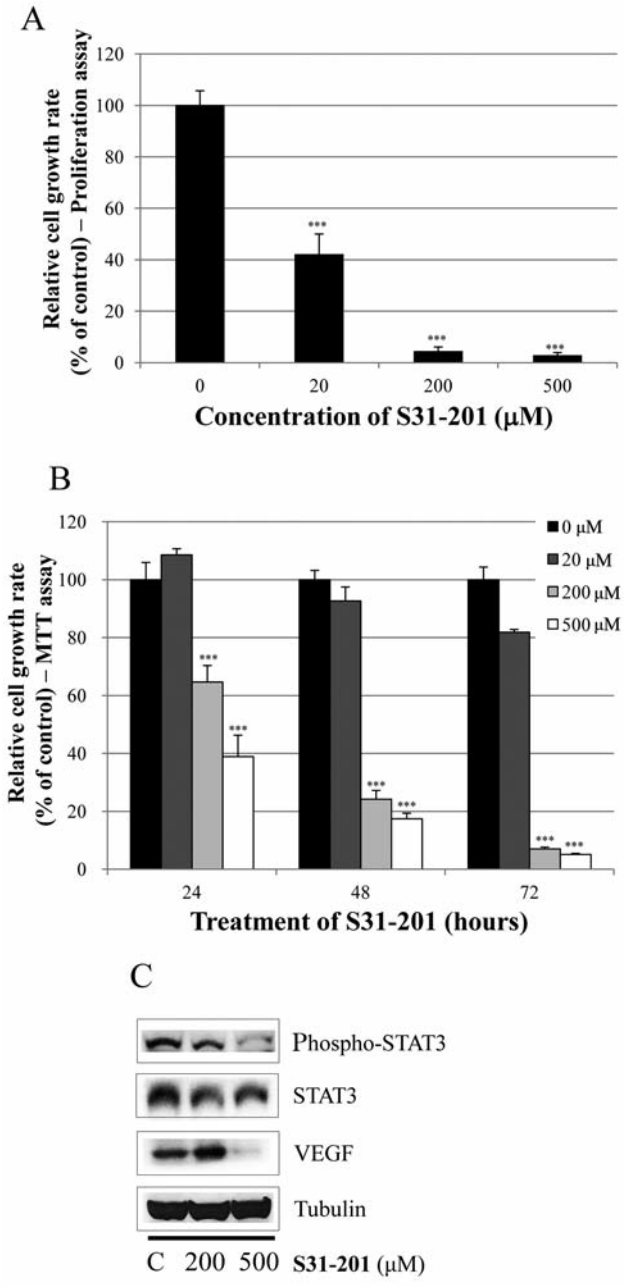

Figure 7. *Effect of the STAT3 inhibitor S31-201 on the growth of MDA-MB-453 cells. (A) MDA-MB-453 cells were treated with different doses of the STAT3 inhibitor S31-201 (0-500 μM). After 72 h, the cell viability was assessed using a cell proliferation assay. (B) MDA-MB-453 cells were treated with different doses of the STAT3 inhibitor S31-201 (0-500 μM). The relative cell growth rate was measured by the MTT assay after 24 h, 48 h and 72 h. The growth rate of the vehicle-treated cells was set to 100%, and the relative decrease in the cell viability resulting from the S31-201 treatment was expressed as a percentage of the control. (C) MDA-MB-453 cells were treated with the STAT3 inhibitor S31-201 for 24 h. Whole-cell lysates were analyzed by western blotting with anti-p-STAT3, anti-STAT3, anti-VEGF, and anti-tubulin antibodies. The data shown are representative of three independent experiments that gave similar results.*

that apigenin decreased the nuclear localization of STAT3 in the presence and absence of $CoCl_2$ (Figure 5D). As seen in Figure 6A, apigenin decreased mRNA levels of *HIF-1α* and *VEGF* that was slightly up-regulated by $CoCl_2$. Moreover, apigenin strongly decreased the $CoCl_2$-induced up-regulation of intracellular VEGF (Figure 6B). These results suggest that apigenin decreases the cell growth rate by inhibiting the JAK2-STAT3-VEGF signaling pathway.

Effect of S31-201 on STAT3 activation in MDA-MB-453 cells. Finally, we investigated whether the STAT3 inhibitor S31-201 inhibits cell proliferation and STAT3 activation in MDA-MB-453 cells. As shown in Figure 7A and B, S31-201 decreased cell growth in a dose- and time-dependent manner. Furthermore, S31-201 reduced the expression of p-STAT3 and VEGF (Figure 7C). These results demonstrate that STAT3 inhibition induces cell growth inhibition and represses the expression of oncogenic molecules.

Discussion

In the present study, we investigated the anti-proliferative activity of apigenin and its mechanisms of action in HER2-overexpressing breast cancer cells (Figure 8). Apigenin suppressed the growth of MDA-MB-453 cells in a dose- (0-100 μM) and time- (0-72 h) dependent manner. The growth inhibition induced by apigenin was related to an increase in the sub-G_0/G_1 apoptotic population in MDA-MB-453 cells. Apigenin increased the number of apoptotic cells in a dose-dependent manner, as assessed by FACS analysis. Interestingly, apigenin induced apoptosis *via* a caspase-dependent pathway by enhancing the cleavage of caspase-8, caspase-3, and PARP. To confirm whether the apoptosis induced by apigenin occurs *via* a caspase-8-, and caspase-3-dependent pathway, we treated MDA-MB-453 cells with the caspase-8 inhibitor Z-IETD-*fmk* and the caspase-9 inhibitor Z-LEHD-*fmk* and performed western blot analysis. We found that the cleavage of caspase-8, caspase-3, and PARP was inhibited by the caspase-8 and caspase-9 inhibitors (Figure 3B). However, this inhibition was abrogated by apigenin, suggesting that this phytochemical contains a strong apoptotic capacity. The caspases, a family of cysteine-dependent aspartate-directed proteases, are common death proteases (32). Caspases are synthesized as relatively inactive zymogens that become activated by scaffold-mediated transactivation or by cleavage *via* upstream proteases in an intracellular cascade (32). Once activated, they cleave a variety of intracellular polypeptides, including major structural elements of the cytoplasm and nucleus, components of the DNA repair machinery, and a number of protein kinases (32).

It should be noted that apigenin did not induce apoptosis *via* the intrinsic mitochondrial apoptosis pathway since this

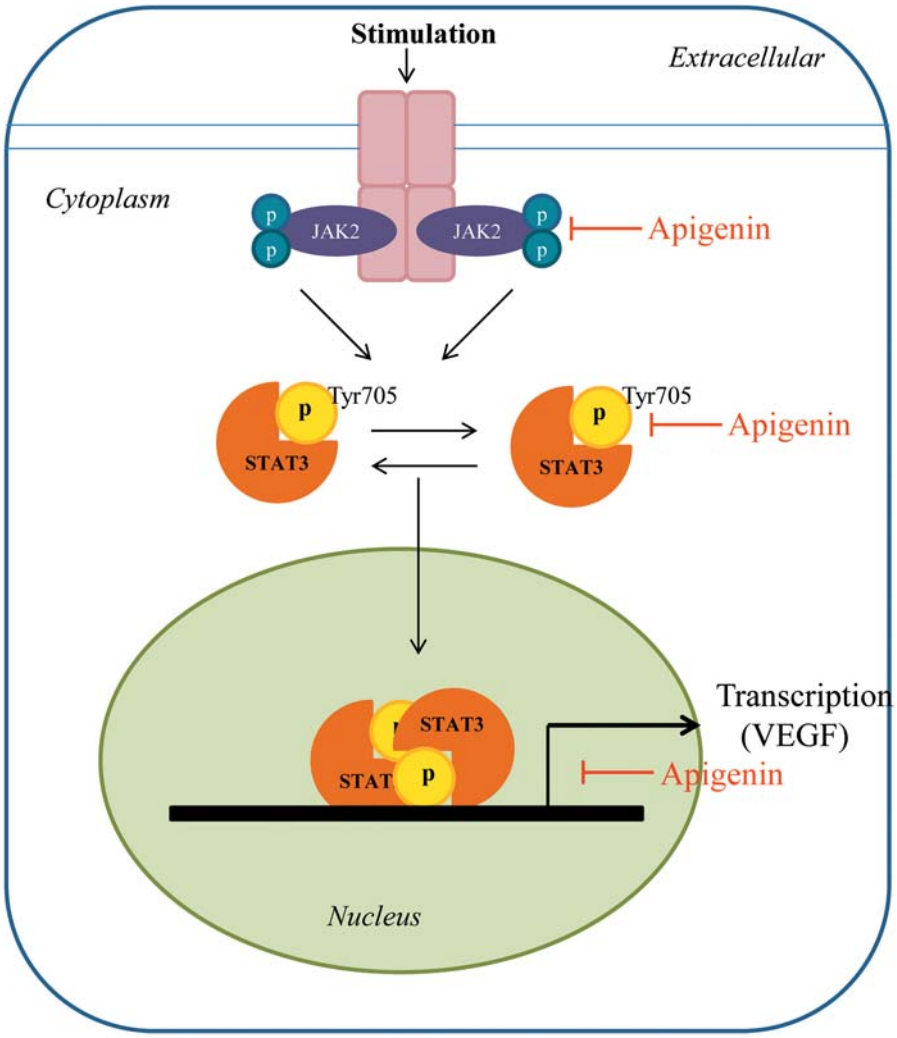

Figure 8. *Molecular mechanism of action for apigenin.*

compound did not reduce the mitochondrial membrane potential, maintaining red flouorescence, and did not affect the levels of Bcl-2 and BAX. BCL2 family includes both anti-apoptotic proteins (Bcl-2, Bcl-XL, MCL-1, and CED-9 etc) and pro-apoptotic proteins (BAX, BAK, DIVA, BCL-Xs, BIK, and BIM *etc*.) (33). All BCL2 members possess at least one of four conserved motifs known as BCL2 homology domains (BH1 to BH4) (33). Pro- and anti-apoptotic family members can heterodimerize and regulate each other's functions; their relative concentration may be important in modulating apoptosis (33). On the other hand, apigenin induced the extrinsic apoptosis pathway: apigenin activated death receptor-related apoptosis signaling, enhancing the cleavage of caspase-8, caspase-3 and PARP.

Our western blot data also indicated that apigenin slightly increased the expression of p53, active p53 (p-p53) and p21, suggesting that this compound suppresses HER2-overexpressing cancer cell growth *via* a p53-dependent manner. In agreement with our data, apigenin has been shown to induce G_1 or G_2/M arrest and apoptosis in human prostate carcinoma cells (34, 35), human cervical carcinoma cells (36), and human hepatoma cells (37) through a p53-dependent manner. The p53 tumor suppressor inhibits cellular proliferation by inducing cell-cycle arrest and apoptosis in response to cellular stresses including DNA damage, growth factor deprivation, hypoxia, and oncogene activation (38, 39). p53-dependent apoptosis is produced by the caspase proteinases and is related to pro-apoptotic proteins such as BAX, NOXA and PUMA (38).

Interestingly, apigenin reduced the expression of p-STAT3 and p-JAK2 in MDA-MB-453 cells. The VEGF promoter contains various transcription factor binding sites, including sites for STAT3 (40) and HIF-1 (41). The physical interaction

of STAT3 with HIF-1 controls VEGF transcriptional activation by their binding to the VEGF promoter (42). In our study, we found that apigenin inhibited VEGF expression/production as well as STAT3 expression/nuclear localization in the presence or absence of $CoCl_2$. The STAT3 inhibitor S31-201 decreased the expression of p-STAT3 and VEGF. In our experiments, culturing of MDA-MB-453 cells under conditions that mimicked hypoxia or normoxia did not induce the expression or activation of MMP-2 or MMP-9 (data not shown), as indicated in a previous report (43). The co-culture of MDA-MB-453 with another cell line or tumor-associated macrophages induces the expression and activation of MMP-2 and MMP-9 (43). Our data clearly demonstrate that apigenin suppresses cell growth by inhibiting the STAT3-VEGF signaling pathway in MDA-MB-453 cells (Figure 8). STAT3 is a transcription factor that regulates the gene expression in response to various cellular stimuli and plays an important role in cell growth and apoptosis. STAT3 usually acts as a tumor promoter, although its role as a tumor-suppressor has been recently reported (44, 45). STAT3 accelerates cell proliferation and angiogenesis, inhibits apoptosis, and drives invasion and metastasis (36-38). STAT3 in melanoma tumors is associated with poor prognosis (46-48). Constitutive STAT3 phosphorylation is mediated by several upstream kinases (Jak and Src) and is thought to be a key component of the oncogenic process (49, 50). Another phytoestrogen (resveratrol) is known to inhibit STAT3 signaling and induces the apoptosis of malignant cells containing activated STAT3 (51).

Approximately 20-25% of invasive breast carcinomas show HER2 overexpression (52). A normal breast cell has 20,000 HER2 receptors, but a breast cancer cell could have up to 1.5 million. HER2 is a member of the HER/ErbB2/Neu protein family, which also includes HER1/EGFR, HER3 and HER4. HER2 cross-talks with the estrogen receptor (ER) signal transduction pathway (53), and its expression levels can be regulated by ER. In our study, we found that apigenin significantly inhibited the growth of HER2-overexpressing cancer cells. This indicates that apigenin could be a useful natural therapy that inhibits HER2-overexpressing breast cancer. Apigenin could be a promising target for the treatment and prevention of HER2-overexpressing breast cancer.

Declaration of Interest

The Authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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HYE SOOK SEO performed most of experiments and wrote the manuscript. JIN MO KU performed RT-PCR and Elisa assay. HAN SEOK CHOI performed FACS analysis. JONG-KYU WOO, BO-HYOUNG JANG and YONG CHEOL SHIN advised the discussion. SEONG-GYU KO designed, supervised the experiments and corrected the manuscript.

References

1 Patel D, Shukla S and Gupta S: Apigenin and cancer chemoprevention: progress, potential and promise (review). Int J Oncol *30*: 233-245, 2007.

2 Shukla S and Gupta S: Apigenin: A Promising Molecule for Cancer Prevention. Pharmaceutical Res *27*: 962-978, 2010

3 Rezai-Zadeh K, Ehrhart J, Bai Y, Sanberg PR, Bickford P, Tan J and Shytle RD: Apigenin and luteolin modulate microglial activation *via* inhibition of STAT1-induced CD40 expression. J Neuroinflammation *5*: 41, 2008.

4 Moon DO, Kim MO, Choi YH, Lee HG, Kim ND and Kim GY: Gossypol suppresses telomerase activity in human leukemia cells *via* regulating hTERT. FEBS Lett *582*: 367-373, 2008.

5 Nakazawa T, Yasuda T, Ueda J and Ohsawa K: Antidepressant-like effects of apigenin and 2,4,5-trimethoxycinnamic acid from Perilla frutescens in the forced swimming test. Biol Pharm Bull *26*: 474-480, 2003.

6 Shukla S and Gupta S: Molecular targets for apigenin-induced cell cycle arrest and apoptosis in prostate cancer cell xenograft. Mol Cancer Ther *5*: 843-852, 2006.

7 Liang YC, Huang YT, Tsai SH, Lin-Shiau SY, Chen CF and Lin JK: Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages. Carcinogenesis *20*: 1945-1952, 1999.

8 Birt DF, Mitchell D, Gold B, Pour P and Pinch HC: Inhibition of ultraviolet light induced skin carcinogenesis in SKH-1 mice by apigenin, a plant flavonoid. Anticancer Res *17*: 85-91, 1997.

9 Seo HS, Ju JH, Jang K and Shin I: Induction of apoptotic cell death by phytoestrogens by up-regulating the levels of phospho-p53 and p21 in normal and malignant estrogen receptor α-negative breast cells. Nutrition Res *31*: 139-146, 2011.

10 Lu HF, Chie YJ, Yang MS, Lee CS, Fu JJ, Yang JS, Tan TW, Wu SH, Ma YS, Ip SW and Chung JG: Apigenin induces caspase-dependent apoptosis in human lung cancer A549 cells through Bax- and Bcl-2-triggered mitochondrial pathway. Int J Oncol *36*: 1477-1484, 2010.

11 Wang W, Heideman L, Chung CS, Pelling JC, Koehler KJ and Birt DF: Cell-cycle arrest at G2/M and growth inhibition by apigenin in human colon carcinoma cell lines. Mol Carcinog *28*: 102-110, 2000.

12 Turktekin M, Konac E, Onen HI, Alp E, Yilmaz A and Menevse S: Evaluation of the effects of the flavonoid apigenin on apoptotic pathway gene expression on the colon cancer cell line (HT29). J Med Food *14*: 1107-1117, 2011.

13 Gupta S, Afaq F and Mukhtar H: Involvement of nuclear factor-kappa B, Bax and Bcl-2 in induction of cell cycle arrest and apoptosis by apigenin in human prostate carcinoma cells. Oncogene *21*: 3727-3738, 2002.

14 Ruela-de-Sousa RR, Fuhler GM, Blom N, Ferreira CV, Aoyama H and Peppelenbosch MP: Cytotoxicity of apigenin on leukemia cell lines: implications for prevention and therapy. Cell Death Dis *1*: e19, 2010.

15 Ujiki MB, Ding XZ, Salabat MR, Bentrem DJ, Golkar L, Milam B, Talamonti MS, Bell RH Hr, Iwamura T and Adrian TE: Apigenin inhibits pancreatic cancer cell proliferation through G2/M cell cycle arrest. Mol Cancer *5*: 76, 2006.
16 Fan TJ, Han LH, Cong RS and Liang J: Caspase family proteases and apoptosis. Acta Biochim Biophys Sin (Shanghai) *37*: 719-727, 2005.
17 Bosch M, Poulter NS, Vatovec S and Franklin-Ton VE: Initiation of programmed cell death in self-incompatibility: role for cytoskeleton modifications and several caspase-like activities. Mol Plant *1*: 879-887, 2008.
18 Mukhopadhyay S, Panda PK, Sinha N, Das DN and Bhutia SK: Autophagy and apoptosis: where do they meet? Apoptosis 2014. [Epub ahead of print].
19 Zhang A, Wu Y, Lai HWL and Yew DT: Apoptosis – a brief review. Neuroembryology *3*: 47-59, 2004.
20 Waring P and Mullbacher A: Cell death induced by the Fas/Fas ligand pathway and its role in pathology. Immunol Cell Biol *77*: 312-317, 1999.
21 Gupta S: Molecular signaling in death receptor and mitochondrial pathways of apoptosis (review). Int J Oncol *22*: 15-20, 2003.
22 Green DR and Reed JC: Mitochondria and apoptosis. Science *281*: 1309-1312, 1998.
23 Boulares AH, Yakovlev AG, Ivanova V, Stoica BA, Wang G, Iyer S and Smulson M: Role of poly(ADP-ribose) polymerase (PARP) cleavage in apoptosis. Caspase 3-resistant PARP mutant increases rates of apoptosis in transfected cells. J Biol Chem *274*: 22932-22940, 1999.
24 Rouanet P, Roger P, Rousseau E, Thibault S, Romieu G, Mathieu A, Cretin J, Barneon G, Granier M, Maran-Gonzalez A, Daures JP, Boissiere F and Bibeau F: HER2 overexpression a major risk factor for recurrence in pT1a-bN0M0 breast cancer: results from a French regional cohort. Cancer Med 2014. doi: 10.1002/cam4.167. [Epub ahead of print]
25 Wilson CA, Cajulis EE, Green JL, Olsen TM, Chung YA, Damore MA, Dering J, Calzone FJ and Slamon DJ: HER-2 overexpression differentially alters transforming growth factor-beta responses in luminal *versus* mesenchymal human breast cancer cells. Breast Cancer Res *7*: R1058-1079, 2005.
26 Joshi JP, Brown NE, Griner SE and Nahta R: Growth differentiation factor 15 (GDF15)-mediated HER2 phosphorylation reduces trastuzumab sensitivity of HER2-overexpressing breast cancer cells. Biochem Pharmacol *82*: 1090-1099, 2011.
27 Favoni RE, Daga A, Malatesta P and Florio T: Preclinical studies identify novel targeted pharmacological strategies for treatment of human malignant pleural mesothelioma. Br J Pharmacol *166*: 532-553, 2012.
28 Tokunaga E, Oki E, Nishida K, Koga T, Egashira A, Morita M, Kakeji Y and Maehara Y: Trastuzumab and breast cancer: Developments and current status. Int J Clin Oncol *11*: 199-208, 2006.
29 Dean-Colomb W and Esteva FJ: Her2-positive breast cancer: Herceptin and beyond. Eur J Cancer *44*: 2806-2812, 2008.
30 Seo HS, Choi HS, Choi HS, Choi YK, Um JY, Choi I, Shin YC and Ko SG: Phytoestrogens induce apoptosis *via* extrinsic pathway, inhibiting nuclear factor-kappaB signaling in HER2-overexpressing breast cancer cells. Anticancer Res *31*: 3301-3313. 2011.
31 Seo HS, Choi HS, Kim SR, Choi YK, Woo SM, Shin I, Woo JK, Park SY, Shin YC and Ko SG: Apigenin induces apoptosis *via* extrinsic pathway, inducing p53 and inhibiting STAT3 and NFκB signaling in HER2-overexpressing breast cancer cells. Mol Cell Biochem *366*: 319-334, 2012.
32 Earnshaw WC, Martins LM and Kaufmann SH: Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem 68: 383-424, 1999.
33 Adams JM and Cory S: The bcl-2 protein family: Arbiters of cell survival. Science *281*: 1322-1326, 1998.
34 Gupta S, Afaq F and Mukhtar H: Involvement of nuclear factor-kappa B, Bax and Bcl-2 in induction of cell cycle arrest and apoptosis by apigenin in human prostate carcinoma cells. Oncogene *21*: 3727-3738, 2002.
35 Shukla S and Gupta S: Molecular targets for apigenin-induced cell cycle arrest and apoptosis in prostate cancer cell xenograft. Mol Cancer Ther *5*: 843-852, 2006.
36 Zheng PW, Chiang LC and Lin CC: Apigenin induced apoptosis through p53-dependent pathway in human cervical carcinoma cells. Life Sci *76*: 1367-1379, 2005.
37 Chiang LC, Ng LT, Lin IC, Kuo PL, and Lin CC: Anti-proliferative effect of apigenin and its apoptotic induction in human Hep G2 cells. Cancer Lett *237*: 207-214, 2006.
38 Schuler M and Green DR: Mechanisms of p53-dependent apoptosis. Biochem Soc T *29*: 684-688, 2001.
39 Shen Y and White E: P53-dependent apoptosis pathways. Adv Cancer Res *82*: 55-84, 2001.
40 Niu G, Wright KL, Huang M, Song L, Haura E, Turkson J, Zhang S, Wang T, Sinibaldi D, Coppola D, Heller R, Ellis LM, Karras J, Bromberg J, Pardoll D, Jove R, and Yu H: Constitutive Stat3 activity up-regulates VEGF expression and tumor angiogenesis. Oncogene *21*: 2000-2008, 2002.
41 Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD and Semenza GL: Activation of vascular endothelial growth factor gene transcription by Hypoxia-inducible factor 1. Mol Cell Biol *16*: 4604-4613, 1996.
42 Jung JE, Lee HG, Cho IH, Chung DH, Yoon SH, Yang YM, Lee JW, Choi S, Park JW, Ye SK and Chung MH: STAT3 is a potential modulator of HIF-1-mediated VEGF expression in human renal carcinoma cells. FASEB J *19*: 1296-1298, 2005.
43 Hagemann T, Robinson SC, Schulz M, Trümper L, Balkwill FR and Binder C: Enhanced invasiveness of breast cancer cell lines upon co-cultivation with macrophages is due to TNF-alpha dependent up-regulation of matrix metalloproteases. Carcinogenesis *25*: 1543-1549, 2004.
44 de la Iglesia N, Konopka G, ram SV, Chan JA, Bachoo RM, You MJ, Levy DE, Depinho RA and Bonni A: Identification of a PTEN-regulated STAT3 brain tumor suppressor pathway. Genes & Develop *22*: 449-462, 2008.
45 Lewis HD, Winter A, Murphy TF, Tripathi S, Pandey VN and Barton BE: STAT3 inhibition in prostate and pancreatic cancer lines by STAT3 binding sequence oligonucleotides: Differential activity between 5' and 3' ends. Mol Cancer Ther *7*: 1543-1550, 2008.
46 Kortylewski M, Jove R and Yu H: Targeting STAT3 affects melanoma on multiple fronts. Cancer Metastasis Rev *24*: 315-327, 2005.
47 Niu G, Bowman T, Huang M, Shivers S, Reintgen D, Daud A, Chang A, Kraker A, Jove R and Yu H: Roles of activated Src and Stat3 signaling in melanoma tumor cell growth. Oncogene *21*: 7001-7010, 2002.

48 Xie TX, Huang FJ, Aldape KD, Kang SH, Liu M, Gershenwald JE, Xie K, Sawaya R and Huang S: Activation of stat3 in human melanoma promotes brain metastasis. Cancer Res *66*: 3188-3196, 2006.

49 Sellers LA, Feniuk W, Humphrey PP and Lauder H: Activated G proteincoupled receptor induces tyrosine phosphorylation of STAT3 and agonist-selective serine phosphorylation *via* sustained stimulation of mitogen-activated protein kinase. Resultant effects on cell proliferation. J Biol Chem *274*: 16423-16430, 1999.

50 Zhang Y, Turkson J, Carter-Su C, Smithgall T, Levitzki A, Kraker A, Krolewski JJ, Medveczky P and Jove R: Activation of Stat3 in v-Src-transformed fibroblasts requires cooperation of Jak1 kinase activity. J Biol Chem *275*: 24935-24944, 2000.

51 Kotha A, Sekharam M, Cilenti L, Siddiquee K, Khaled A, Zervos AS, Carter B, Turkson J and Jove R: Resveratrol inhibits src and Stat3 signaling and induces the apoptosis of malignant cells containing activated Stat3 protein. Mol Cancer Ther *5*: 621-629, 2006.

52 Tolaney SM and Krop IE: Mechanisms of trastuzumab resistance in breast cancer. Anticancer Agents Med Chem *9*: 348-355, 2009.

53 Buzdar AU: Role of biologic therapy and chemotherapy in hormone receptor- and HER2-positive breast cancer. Ann Oncol *20*: 993-999, 2009.

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