

# Effects of apicidin, a histone deacetylase inhibitor, on the regulation of apoptosis in H-*ras*-transformed breast epithelial cells

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**Abstract.** The cellular susceptibility of cancer cells to histone deacetylase (HDAC) inhibitors is increased by the ectopic expression of oncogenic Ras. However, the ability of HDAC inhibitors to regulate the apoptotic pathway in human breast cancer cells is still not completely understood. In this study, the anti-proliferative effects of apicidin were compared in H-*ras*-transformed human breast epithelial (MCF10A-*ras*) and non-transformed epithelial (MCF10A) cells. MCF10A-*ras* cells showed a significantly higher growth rate than MCF10A cells. Apicidin significantly increased the levels of acetylated histone H3 and H4 in both cell lines. Western blot analysis and flow cytometry were used to determine if the anti-proliferative effects of apicidin in MCF10A and MCF10A-*ras* cells could be mediated by modulating the cell cycle. Apicidin attenuated the expression of cyclin E and CDK2 in MCF10A cells, decreased cyclin D1 and cyclin E levels in MCF10A-*ras* cells, and increased the levels of CDK inhibitors, p21<sup>WAF1/Cip1</sup> and p27<sup>Kip1</sup>, in both cell lines. Notably, the levels of hyperphosphorylation of the Rb protein levels were lower in the MCF10A-*ras* cells after apicidin treatment. Studies on the regulation of apoptosis showed that apicidin induces the up-regulation of p53 and the downstream activation of ERK in MCF10A-*ras* cells. The up-regulation of p53 promoted Bax expression leading to activation of caspases-9 and -6, and eventually to apoptosis in MCF10A-*ras* cells. In addition, apicidin significantly increased the levels of ERK1/2 phosphorylation in MCF10A-*ras* cells. Therefore, the apicidin-mediated ERK pathway appears to play an important role in modulating the pro-apoptotic pathway in MCF10A-*ras* cells.

## Introduction

Ras proteins play a key role in controlling the activity of several essential signaling pathways that regulate normal cellular proliferation (1,2). In general, the Ras proteins are activated by a point mutation and are expressed quite frequently in human tumor tissues. The activated Ras protein contributes to several aspects of the malignant phenotype, including the deregulation of tumor cell growth, programmed cell death, invasiveness, and angiogenesis (3). Although *ras* mutations are rarely observed in breast cancers (<5%), there is considerable experimental evidence showing that aberrant *ras*-mediated signaling may promote the development of breast cancer (4).

The Raf serine/threonine kinases are the most well-characterized downstream effector targets for Ras. Ras binds to the Raf proteins, which contributes to their activation. Activated Raf, in turn, phosphorylates and activates mitogen-activated protein kinases 1 and 2 (MEK1/2), which then phosphorylate and activate the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinases 1 and 2 (ERK1/2) (1-3). ERK1/2, once activated, phosphorylates various downstream substrates involved in a variety of cellular responses such as cell proliferation, cell differentiation, cell survival, and cell motility (5). Moreover, the constant activation of the downstream signaling pathways by oncogenic Ras can generate an aberrantly regulated complex signaling network, which can possibly cause the *ras*-transformed cells to show a different susceptibility from their normal counterpart cells to various agents that cause cytotoxicity and apoptosis (6).

Recently, it was reported that a culture of Ras-transformed cells in the presence of the histone deacetylase (HDAC) inhibitor, trichostatin A (TSA), exhibited morphological reversion and cell cycle arrest in the G<sub>1</sub> phase (7). Therefore, HDAC inhibitors might alter the cell morphology of various oncogene-transformed cells and increase the cell cycle arrest in transformed cells (8,9). There is increasing evidence suggesting that HDAC inhibitors modulate the expression of cell cycle-progression and apoptosis-regulated genes, preferably in tumor cells to inhibit proliferation and induce apoptotic cell death (10). Although little is known about the cellular effects of HDAC inhibitors in oncogene-transformed mammalian cells, they are considered to be a novel class of anticancer agents (11,12). Based on these results, it is

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important to understand the Ras-mediated signaling cascade that leads to the increased cell susceptibility to HDAC inhibitors in *ras*-transformed cells. In this study, stably H-*ras*-transformed human breast epithelial (MCF10A-*ras*) cell lines were used as target cells to examine the Ras-mediated apoptotic pathways after exposure to apicidin [cyclo(*N*-O-methyl-L-tryptophanyl-L-isoleuciny-L-D-pipecolinyl-L-2-amino-8-oxodecanoyl)] (structure shown in Fig. 1A). Apicidin is a novel cyclic tetrapeptide with potent broad-spectrum anti-protozoal activity against apicomplexan parasites and anti-proliferative activity against various cancer cell lines (13,14). However, the precise mechanism of how apicidin regulates apoptosis in *ras*-activated human breast epithelial cells is not completely understood. Therefore, this study examined the antitumor effect of apicidin on H-*ras*-transformed cells. The acetylated histone protein and the HDAC protein levels were measured, and the role of apicidin on cell cycle arrest, apoptosis and the expression of the tumor suppressor genes was evaluated.

The results showed that apicidin induces an increase in caspase-9 and -6 levels as well as an increase in ERK1/2 activation in MCF10A-*ras* cells. Overall, activation of the ERK pathway in H-*ras*-transformed cells is clearly associated with both the induction of the anti-proliferative activity through cell cycle arrest at the G<sub>1</sub> phase and the increase in its apoptotic potential through the activation of caspases. This suggests that apicidin has potential use as an HDAC inhibitor for the treatment of breast cancer.

## Materials and methods

**Materials.** Apicidin was obtained from Sigma-Aldrich Biotechnology (St. Louis, MO, USA). Apicidin was re-suspended in dimethyl sulfoxide (DMSO). This chemical was diluted to appropriate concentrations with the culture medium containing 5% charcoal-dextran-treated fetal bovine serum (CD-FBS). The final concentration of DMSO in each treatment was <0.1% (vol/vol). All other chemicals were of the highest quality commercially available.

**Cell cultures.** MCF10A, which is a spontaneously immortalized cell line derived from diploid primary human breast epithelial cells without viral or chemical intervention, and the MCF10A-*ras* cells were kindly provided by Dr A. Moon (Duksung Women's University, Seoul, Korea). MCF10A and MCF10A-*ras* cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 5% horse serum, 0.5 g/ml hydrocortisone, 10 g/ml insulin, 20 ng/ml epidermal growth factor (EGF), 0.1 g/ml cholera enterotoxin, 100 units/ml penicillin-streptomycin, 2.5 mM L-glutamine and 0.5 g/ml fungizone. Retroviral vectors containing a mutant H-*ras* (pBW 1423) were transfected into the amphotropic package cell line Gp+envAm12 (10). MCF10A cells were infected with the viral media and selected in the presence of 400 g/ml G418 (10). More than 100 G418-resistant colonies were pooled together. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were plated on plastic dishes, and the culture medium was replaced every 2 days. For cell growth analysis the cells were cultured at a density of 1x10<sup>4</sup> viable cells in 6-well plates. The cells were then

trypsinized, and the number of viable cells was counted daily over a 6-day period.

**Ras activation assay.** The levels of activated Ras in cell lysates isolated from the control MCF10A and the MCF10A-*ras* cells were determined using a Ras activation assay kit (Upstate, Billerica, MA, USA; catalog no. 17-218) with the horseradish peroxidase-conjugated anti-mouse antibody (Santa Cruz, CA, USA; catalog no. sc-2055) according to the manufacturer's instructions.

**MTT assay.** Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT assay relies primarily on the mitochondrial metabolic capacity of viable cells, and reflects the intracellular redox state. In this method, viable cells reduced the yellow tetrazolium salt (MTT) to the purple formazan. The MCF10A and MCF10A-*ras* cells were cultured in 96-well plates at a density of 2x10<sup>3</sup> cells per well for 48 h. After incubation, the cells were treated with a 5 mg/ml MTT solution at 37°C for 4 h and then dissolved in DMSO. The ratio of viable cells was determined by measuring the absorbance per well at 540 nm using a VersaMax™ Microplate Reader (Molecular Devices Corp., Sunnyvale, CA, USA).

**DAPI staining of nuclei.** The morphological changes in the nuclear chromatin of the cells undergoing apoptosis were detected by staining with the DNA-binding fluorochrome, 4',6-diamidino-2-phenylindole (DAPI). The cells were grown in 6-well plates at a density of 2x10<sup>4</sup> cells per well. After treatment, cells were fixed in absolute methanol and stained with 300 μl of a DAPI solution (1 g/ml) at 37°C for 15 min. The staining solution was removed, and the cells were examined by fluorescence microscopy (Axiovert 200, Carl Zeiss, Jena, Thuringen, Germany).

**Preparation of cell extracts.** The nuclear extracts from the control and apicidin-treated MCF10A and MCF10A-*ras* cells were prepared using the following procedure. All the solutions, tubes, and centrifuges were maintained at 4°C. The cells were harvested by scraping, washed with ice-cold phosphate-buffered saline (PBS), centrifuged for 5 min, and incubated for 20 min in 50 μl of lysis buffer I [10 mM HEPES, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. The crude nuclei released as a result of lysis were collected by microcentrifugation for 10 min and re-suspended for 20 min with lysis buffer II (2.75% lysis buffer I and 0.05% NP-40). After 10 min of microcentrifugation, the pellet was re-suspended in 40 μl of lysis buffer III (5 mM HEPES, pH 7.9, 300 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF and 26% glycerol) for 30 min and clarified by final microcentrifugation for 30 min. The total extracts were prepared using the following procedures. Cells were harvested by scraping and washed with ice-cold PBS. After microcentrifugation, the total proteins were prepared using a Pro-Prep™ protein extract solution (Intron, Seongnam, Korea). The protein concentrations were determined using the protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA).

Table I. Primary and secondary antibodies used in the Western blot analysis.

Antibody	2nd antibody	Dilution ratio	Company
Ac-H3	rabbit	1:5,000	Upstate
Ac-H4	rabbit	1:5,000	Upstate
Histone H1	mouse	1:200	Santa Cruz
Cyclin D1	rabbit	1:200	Santa Cruz
Cyclin E	rabbit	1:200	Santa Cruz
CDK4	rabbit	1:200	Santa Cruz
CDK2	mouse	1:200	Santa Cruz
p53	mouse	1:200	Santa Cruz
p21 <sup>WAF1/Cip1</sup>	mouse	1:200	Santa Cruz
p27 <sup>Kip1</sup>	rabbit	1:1,000	Santa Cruz
Rb	rabbit	1:200	Santa Cruz
p-Rb	rabbit	1:500	Santa Cruz
Caspase-9	rabbit	1:200	Santa Cruz
Caspase-6	rabbit	1:200	Santa Cruz
Caspase-3	rabbit	1:500	Santa Cruz
Bax	mouse	1:200	Santa Cruz
Bcl-2	mouse	1:200	Santa Cruz
c-Jun	rabbit	1:200	Santa Cruz
c-Fos	mouse	1:200	Santa Cruz
$\beta$ -actin	mouse	1:1,000	Santa Cruz
ERK1/2	rabbit	1:200	Cell Signaling
p-ERK1/2	mouse	1:200	Cell Signaling

**Western blot analysis.** Equal amounts of protein extracts were denatured by boiling at 96°C for 5 min in sample buffer (0.5 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.1% bromophenol blue, 10%  $\beta$ -mercaptoethanol). The proteins were subjected to SDS-PAGE on 6-15% slab gels, and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories). The membranes were blocked with 5% non-fat dry milk in a TNT buffer (10 mM Tris-HCl, pH 7.6, 100 mM NaCl and 0.5% Tween-20) for 1 h at room temperature. The membranes were then probed overnight at 4°C with the appropriate primary antibodies (Table I). After washing for 1 h with the TNT buffer, the membranes were incubated with either the horseradish peroxidase-conjugated anti-mouse antibody or anti-rabbit antibody (Santa Cruz, 1:10,000) for 30 min at room temperature. The membranes were washed again with the TNT buffer for 1 h. Antigen-antibody complexes were detected with ECL Plus Western blotting detection reagents (Amersham Biosciences Corp., Little Chalfont, Bucks, UK).

**Flow cytometry analysis.** The treated cells were harvested and washed with 1% bovine serum albumin in PBS before being fixed with 70% ethanol containing 0.5% Tween-20 at 4°C for at least 1 h. The fixed cells were then washed with 1% bovine serum albumin in PBS and suspended in a cold propidium iodide (PI) staining solution (100 g/ml RNase A and 10 g/ml PI in PBS) at 4°C for 40 min. The stained cells were analyzed for their relative DNA content using a flow cytometry system (Beckman Coulter, Fullerton, CA, USA), which is based on measuring the increase in red fluorescence.

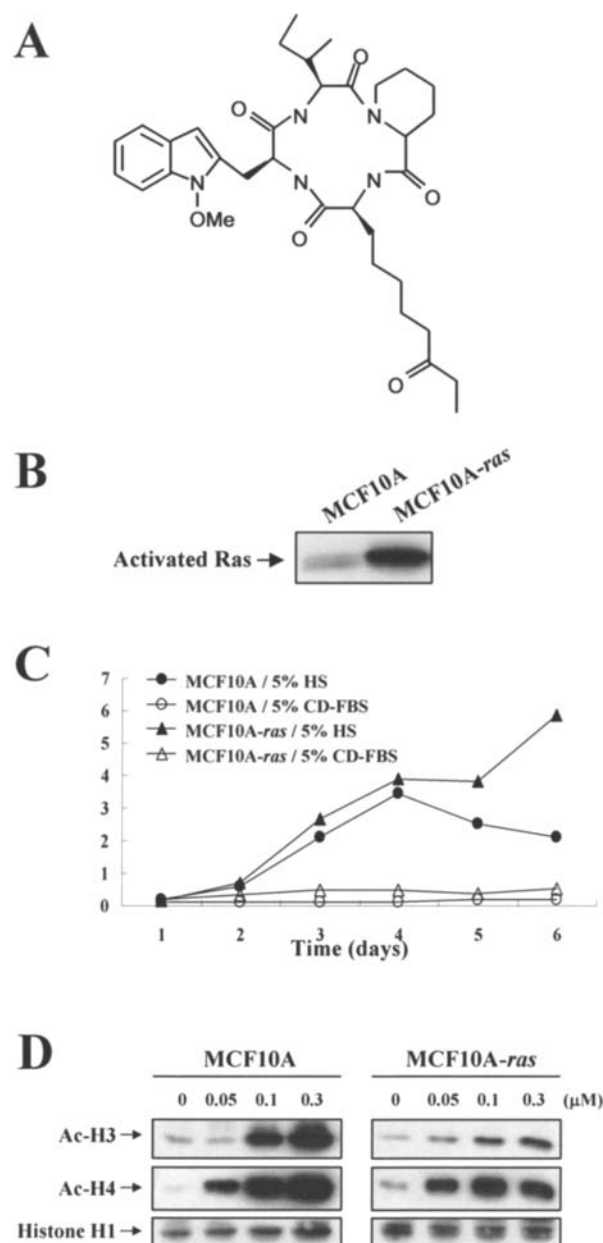


Figure 1. (A) Chemical structure of apicidin. (B) Stable transfection of MCF10A cells with the activated ras oncogene. The levels of activated Ras in the cell lysates of the MCF10A-ras transfectant (MCF10A-ras) and untransfected control (MCF10A) cells were characterized using a Ras activation assay kit. (C) The numbers of MCF10A-ras transfectants and MCF10A cells were determined using a hemocytometer. 5% HS, cells cultured in DMEM/F12 containing 5% horse serum; 5% CD-FBS, cells cultured in DMEM/F12 containing 5% charcoal-dextran-treated fetal bovine serum. (D) The effect of apicidin treatment on acetylation of histone proteins in MCF10A and MCF10A-ras cells. Nuclear extracts were isolated from MCF10A and MCF10A-ras cells treated with apicidin for 24 h. Western blot analysis was carried out using anti-acetyl histone H3 and anti-acetyl histone H4 antibodies. The quantification of the blots for Ac-H3 and Ac-H4 is shown.

**Statistical analysis.** Unless otherwise indicated, all data were expressed as the mean  $\pm$  SD from triplicate experiments performed in a parallel manner. The statistical significance was analyzed using the Student's t-test. The comparisons were determined relative to the untreated controls, and statistical significance was determined as  $p < 0.05$  and  $p < 0.001$ .



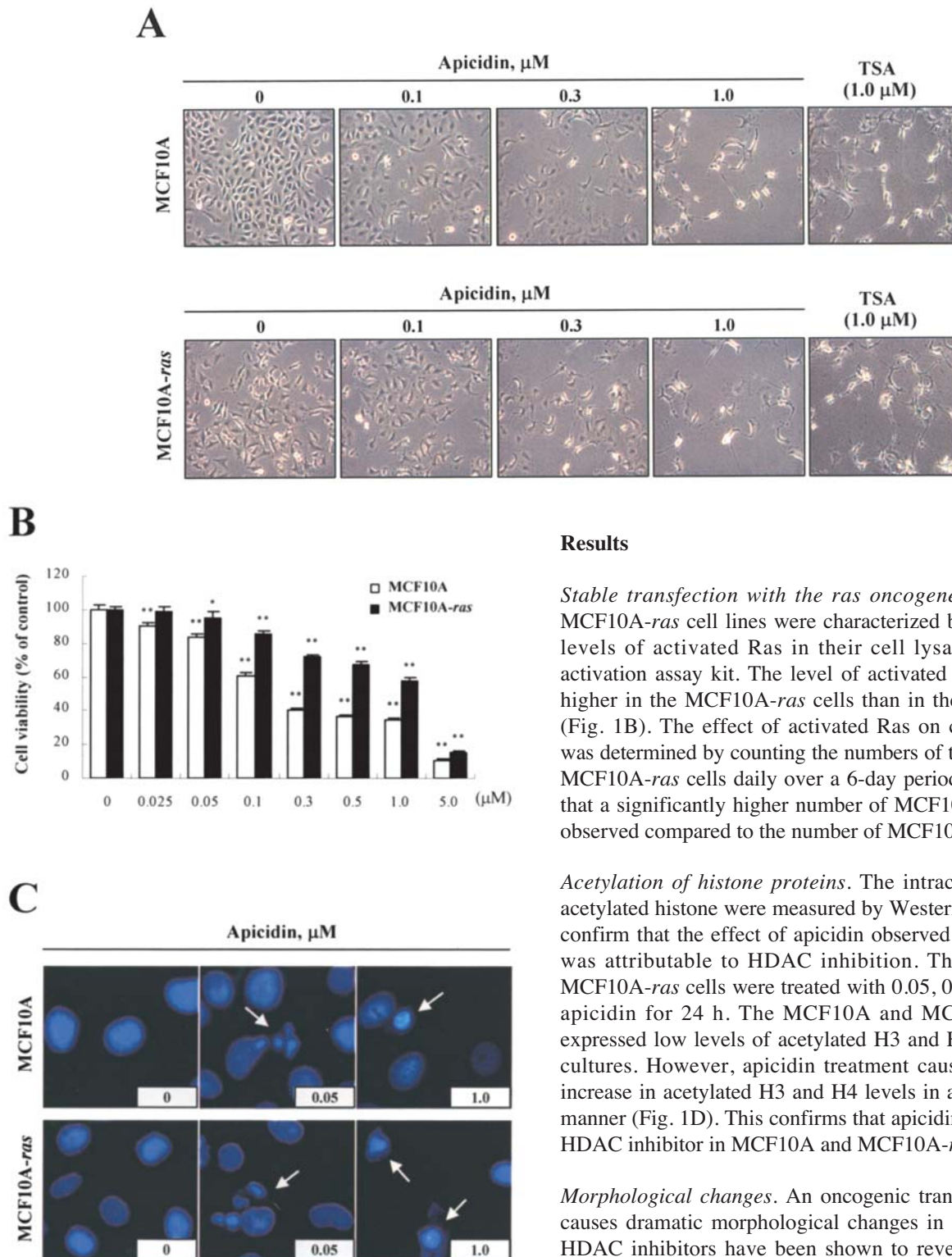


Figure 2. Morphological changes and apoptosis in MCF10A and MCF10A-ras cells induced by apicidin. (A) MCF10A and MCF10A-ras cells were treated for 24 h with the indicated concentrations of apicidin and TSA, and the morphology of the cells was examined microscopically (x200). The morphological changes were induced by apicidin and trichostatin A (TSA). (B) MCF10A and MCF10A-ras cells were treated with different concentrations of apicidin for 48 h. The anti-proliferative effect of apicidin was measured using an MTT assay. (C) MCF10A and MCF10A-ras cells were treated with different concentrations of apicidin and then stained with DAPI. Nuclear morphology was visualized using a fluorescence microscope. Arrows indicate the apoptotic cells with condensed and fragmented nuclei. Data are expressed as the mean  $\pm$  SD. \* $p < 0.05$ ; \*\* $p < 0.001$  compared with the control cells.

## Results

**Stable transfection with the ras oncogene.** MCF10A and MCF10A-ras cell lines were characterized by measuring the levels of activated Ras in their cell lysates using a Ras activation assay kit. The level of activated Ras protein was higher in the MCF10A-ras cells than in the MCF10A cells (Fig. 1B). The effect of activated Ras on cell proliferation was determined by counting the numbers of the MCF10A and MCF10A-ras cells daily over a 6-day period. Fig. 1C shows that a significantly higher number of MCF10A-ras cells was observed compared to the number of MCF10A cells.

**Acetylation of histone proteins.** The intracellular levels of acetylated histone were measured by Western blot analysis to confirm that the effect of apicidin observed in cultured cells was attributable to HDAC inhibition. The MCF10A and MCF10A-ras cells were treated with 0.05, 0.1 and 0.3  $\mu\text{M}$  of apicidin for 24 h. The MCF10A and MCF10A-ras cells expressed low levels of acetylated H3 and H4 in the control cultures. However, apicidin treatment caused a significant increase in acetylated H3 and H4 levels in a dose-dependent manner (Fig. 1D). This confirms that apicidin acts as a potent HDAC inhibitor in MCF10A and MCF10A-ras cells.

**Morphological changes.** An oncogenic transformation often causes dramatic morphological changes in target cells (15). HDAC inhibitors have been shown to reverse the morphological changes in ras-transformed cells (16,17). Therefore, this study examined whether apicidin induces morphological changes in MCF10A and MCF10A-ras cells, respectively. As shown in Fig. 2A, the morphology of MCF10A-ras cells in the culture changed from a rounded morphology to a spindle ras-transformed morphology. Both cell lines showed dramatic changes to an elongated shape with filamentous protrusions after being exposed to apicidin (up to 1.0  $\mu\text{M}$ ). The amount of filamentous protrusions increased in a dose-dependent manner. The morphological changes produced by TSA, another HDAC inhibitor, were similar to those induced by apicidin.

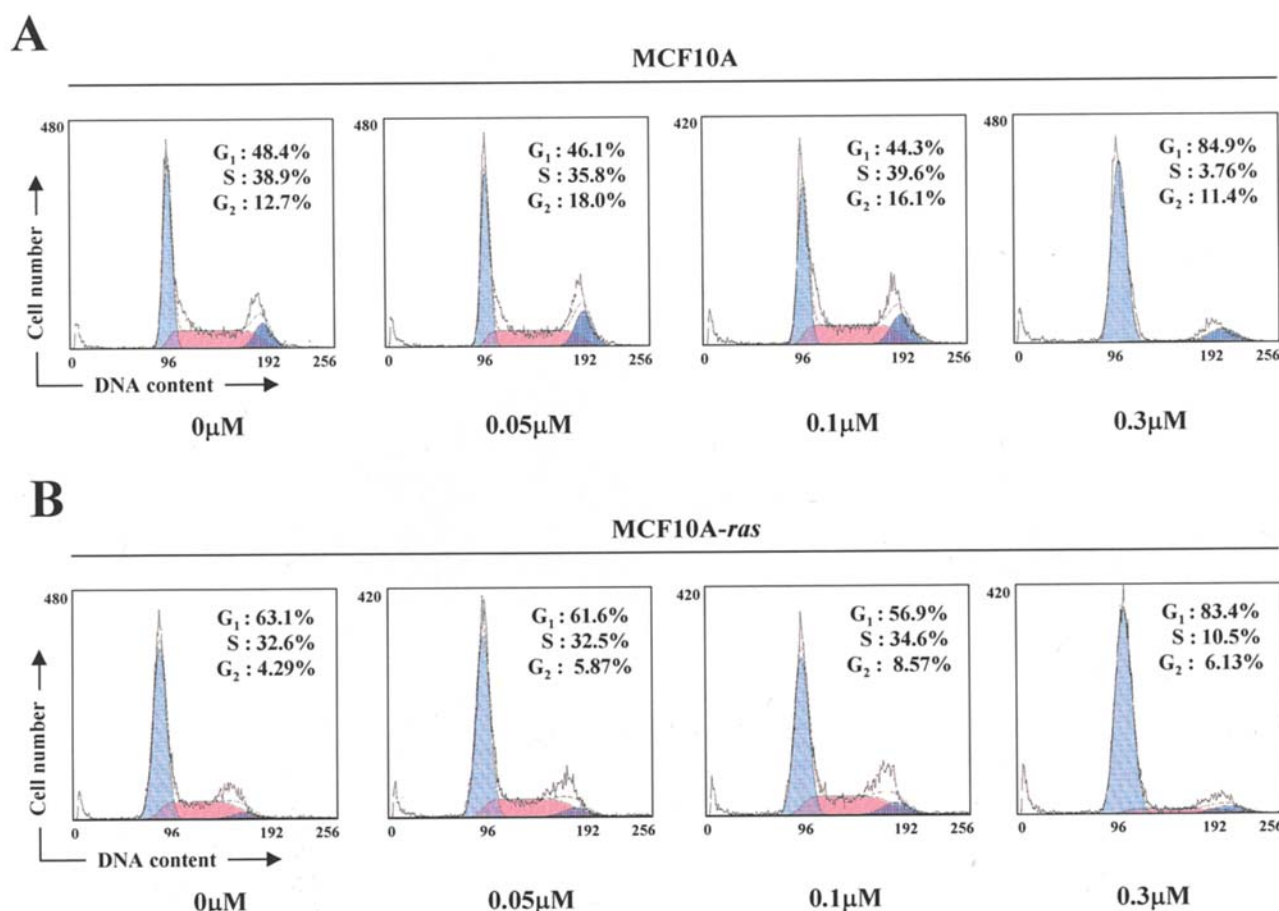


Figure 3. Effect of apicidin on cell cycle distribution. (A) MCF10A and (B) MCF10A-ras cells were treated with different concentrations of apicidin for 24 h. The cell cycle distribution was analyzed by flow cytometry after staining the DNA with propidium iodide (PI).

**Cytotoxicity and apoptotic bodies.** The effect of apicidin on cell proliferation was evaluated using an MTT assay. The MCF10A and MCF10A-ras cells were treated with apicidin at consecutive serial concentrations for 48 h. As shown in Fig. 2B, apicidin inhibited the growth of MCF10A and MCF10A-ras cells in a dose-dependent manner. The IC<sub>50</sub> (50% inhibition concentration) values of apicidin in the MCF10A and MCF10A-ras cells were 1.4 and 0.2 μM, respectively. Hence, the MCF10A-ras cells were more sensitive to the inhibitory effects of apicidin than the MCF10A cells. The MCF10A and MCF10A-ras cells were stained with DAPI, which enters the nuclear membrane and binds to the DNA in order to determine if the decrease in cell viability caused by apicidin was closely related to cellular apoptosis. As shown in Fig. 2C, cellular apoptotic bodies were produced in both cell lines after apicidin treatment for 24 h but not in the control cells. This indicates that apicidin inhibits cell proliferation and causes induction of apoptotic cell death.

**Cell cycle distribution.** Considering that apicidin decreases the levels of cell proliferation, its effect on the cell cycle distribution was also analyzed by flow cytometry analysis. MCF10A and MCF10A-ras cells were treated with 0.05, 0.1 and 0.3 μM apicidin for 24 h. As shown in Fig. 3A, treating the MCF10A cells with 0.3 μM apicidin caused growth arrest in the G<sub>1</sub> phase of the cell cycle (G<sub>1</sub>, 84.9%) compared with

their untreated counterpart cultures. The majority of the surviving cell population in the MCF10A-ras cells after the 0.3-μM apicidin treatment was also growth-arrested in the G<sub>1</sub> phase of the cell cycle (G<sub>1</sub>, 83.4%). Treating the cells with 0.3 μM apicidin caused the accumulation of cells predominantly at the G<sub>1</sub> phase with a concomitant decrease in the number of cells in the S phase (Fig. 3B). Apicidin did alter the sub-G<sub>1</sub> fraction, which suggests that it causes cell cycle arrest with the induction of apoptosis.

**Expression of cell cycle regulator proteins.** The expression levels of the major cell cycle regulators were assessed by Western blot analysis to examine the mechanisms involved in the cell cycle arrest caused by apicidin. The levels of cyclin D1, CDK4, cyclin E, CDK2, p21<sup>WAF1/Cip1</sup>, p27<sup>Kip1</sup> and retinoblastoma (Rb), as well as the level of Rb phosphorylation were determined. Both cyclin D/CDK4 and cyclin E/CDK2 complexes are essential for the cells to progress from the G<sub>1</sub> phase into the S phase. As shown in Fig. 4A, apicidin caused a decrease in cyclin E expression in both cell lines without altering the levels of CDK4. The levels of cyclin D1 were significantly lower in apicidin-treated MCF10A-ras cells than the levels in the MCF10A cells. Apicidin significantly up-regulated the expression of p21<sup>WAF1/Cip1</sup> and p27<sup>Kip1</sup> in both cell lines (Fig. 4B). After exposure to apicidin, there was a significant decrease in the levels of phosphorylated

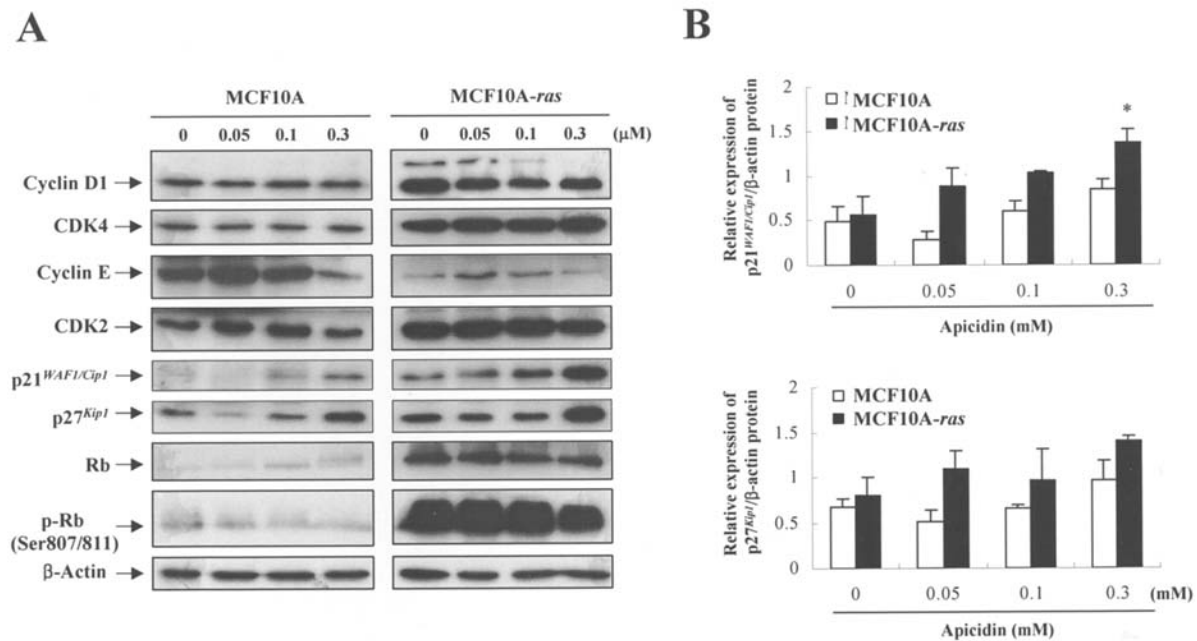


Figure 4. Effect of apicidin on the expression of cell cycle regulatory proteins. MCF10A and MCF10A-ras cells were treated with different concentrations of apicidin for 24 h. Proteins were isolated from MCF10A and MCF10A-ras cells treated with apicidin for 24 h. (A) The expression of cell cycle regulatory proteins was determined by Western blot analysis with the indicated antibodies. (B) The quantification of blots for p21<sup>WAF1/Cip1</sup> and p27<sup>Kip1</sup> is expressed. Autoradiograms were scanned by densitometry. Data are expressed as the mean  $\pm$  SD (n=3). \*p<0.05; \*\*p<0.001 compared with the control cells. CDK, cyclin-dependent kinase; p-Rb, phosphorylated Rb; and Rb, retinoblastoma protein.

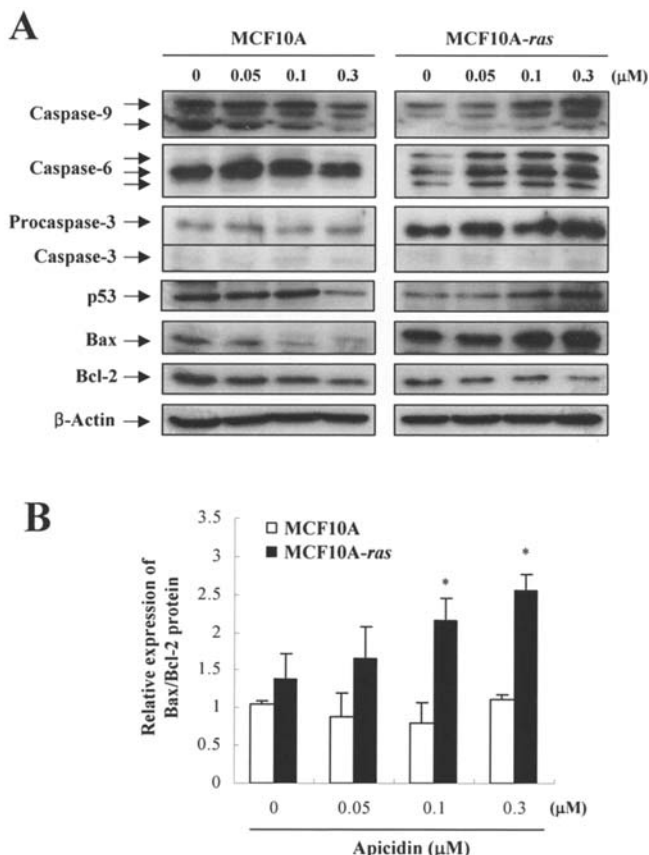


Figure 5. Effect of apicidin on the expression of downstream proteins in the apoptotic pathway. Cells were treated with vehicle alone or with 0.05, 0.1 and 0.3  $\mu$ M of apicidin for 24 h. (A) Western blot analysis was performed using the indicated antibodies. (B) The quantification of the Bax/bcl-2 ratio is expressed. Autoradiograms were scanned by densitometry. Data are expressed as the mean  $\pm$  SD (n=3). \*p<0.05; \*\*p<0.001 compared with the control cells.

Rb (p-Rb) protein in both cell lines. However, the total Rb protein levels in both cell lines were virtually unaffected by apicidin. Therefore, apicidin is likely to block the cell cycle progression from the G<sub>1</sub> to the S phase.

**Expression of downstream proteins in the apoptotic pathway.** Since the results showed that apicidin induces apoptosis in MCF10A and MCF10A-ras cells, this study examined the effect of apicidin on caspases, p53, Bax and Bcl-2, which are regulatory molecules known to induce apoptotic cell death in both cell lines. In the MCF10A-ras cells, apicidin significantly increased the level of active forms of caspases-9 and -6 in a dose-dependent manner. Similarly, p53 protein levels were also significantly higher in the MCF10A-ras cells after apicidin treatment when compared with the MCF10A cells (Fig. 5A). On the other hand, apicidin treatment attenuated caspase-9, caspase-6 and p53 in MCF10A cells (Fig. 5A). Bax and Bcl-2 are homologous proteins that have opposing effects on cell fate, with Bax acting as an accelerator of apoptosis and Bcl-2 serving to prolong cell survival (18). Apicidin treatment increased the levels of Bax expression in MCF10A-ras cells (Fig. 5B). In particular, treatment with 0.3  $\mu$ M apicidin resulted in an 85% increase in the intensity of the band for Bax, whereas no increase in Bax level was observed in MCF10A cells. This suggests that elevated Bax expression levels are involved in the induction of apoptosis in MCF10A-ras cells. This demonstrates that caspases, p53 and Bax play a key role in the apicidin-induced death of MCF10A-ras cells.

**Expression of the H-ras oncogene, ERK1/2, and Ap-1 transcription factors.** The level of ERK1/2 expression in the Ras-signaling pathway was measured in both cell lines after a



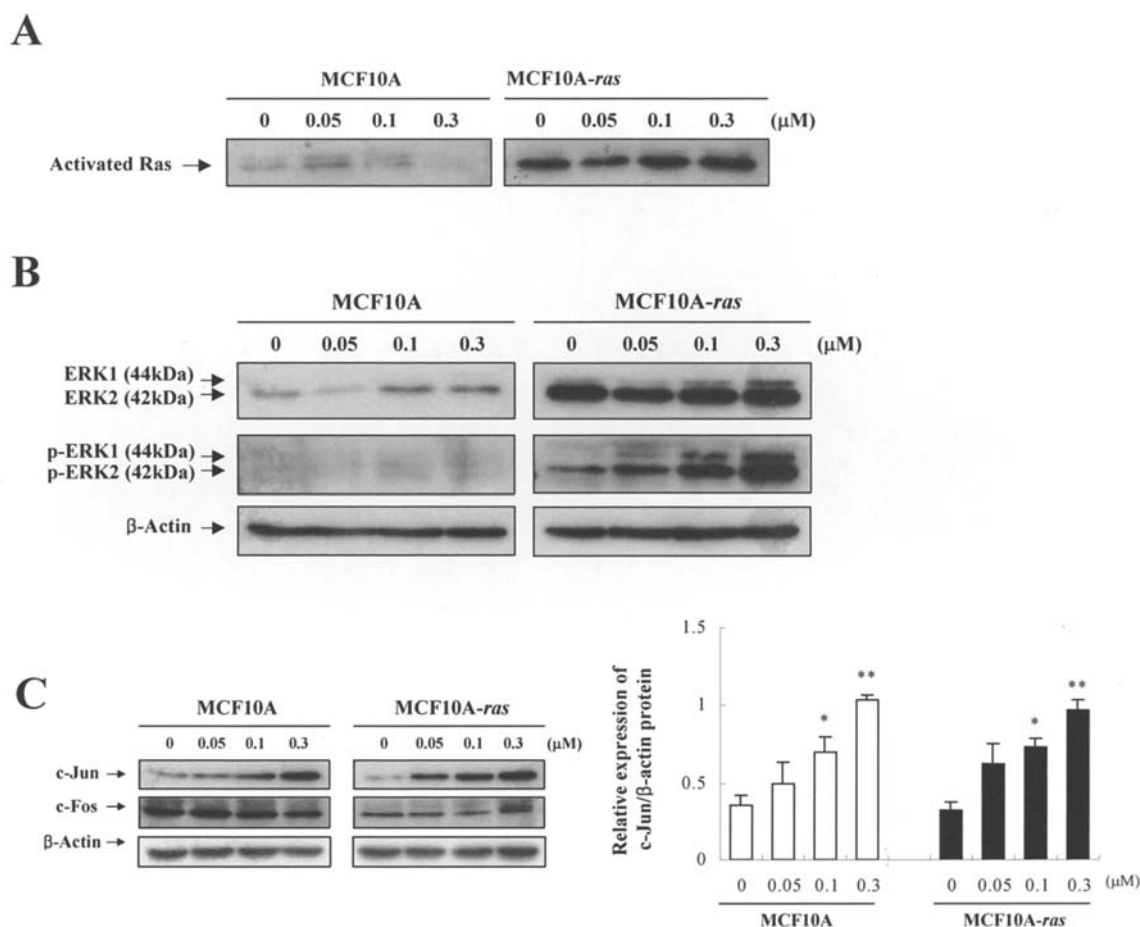


Figure 6. Effect of apicidin on activated Ras protein expression, ERK1/2 activation, and AP-1 transcription factor. Cells were treated with the vehicle alone or with 0.05, 0.1 and 0.3  $\mu$ M of apicidin for 24 h. (A) The levels of Ras protein were determined using a Ras activation assay kit. (B) The protein expression was determined by Western blot analysis with antibodies directed against ERK1/2 and p-ERK1/2. ERK, extracellular signal-regulated kinase; p-ERK, phosphorylated ERK. (C) Western blot analysis was performed using anti-c-Jun and anti-c-Fos antibodies. The relative c-Jun level was assessed by scanning densitometry (bottom panel). Data are expressed as the mean  $\pm$  SD (n=3). \*p<0.05; \*\*p<0.001 as compared with the control cells.

24-h apicidin treatment to further examine the molecular basis for the apicidin-induced cell cycle arrest and apoptosis. As shown in Fig. 6A, the ectopically expressed H-Ras was observed in MCF10A-ras cells after the apicidin treatment but the ectopically activated H-Ras protein was not affected in MCF10A-ras cells. Parallel to the levels of ectopically expressed H-Ras, the activation of the ERK pathway, which is the signaling pathway downstream of Ras, was examined by measuring the level of ERK1/2 phosphorylation (Fig. 6B). Apicidin treatment increased the levels of phosphorylated ERK1/2 in MCF10A-ras cells but not in MCF10A cells. Therefore, the ERK pathway downstream of Ras is involved in mediating the signals that modulate cell cycle arrest and cell death in MCF10A-ras cells. Activator protein-1 (AP-1) transcription factor is a dimeric complex that comprises members of the Jun, Fos, activating transcription factor (ATF), and musculoaponeurotic fibrosarcoma (MAF) protein families (19). A previous *in vitro* study suggested that increased AP-1 activity could lead to apoptosis in specific cell types, including human tumor cells (20). As shown in Fig. 6C, apicidin increased the levels of c-Jun protein in MCF10A and MCF10A-ras cells. However, the expression levels of c-Fos were not changed after apicidin treatment.

## Discussion

The malignant transformation of oncogenic Ras proteins leads to a cellular transformation and promotes tumorigenesis through molecular and cellular changes (20). These changes alter the susceptibility of transformed cells to antitumor agents compared with their normal counterpart cells. There is considerable evidence suggesting that the HDAC inhibitors alter the cellular morphology and cause cell cycle arrest in oncogene-transformed cells or various cancer cells (21,22). However, the molecular mechanisms for the effects of HDAC inhibitors on the regulation of apoptosis through the Ras-activated ERK pathways are not clearly understood. Several reports have shown that *ras*-transformed cells have a higher susceptibility to anticancer agents, such as etoposide (23) and depsipeptide FR901228 (24).

The results from this study clearly show that apicidin produces morphological changes and induces cell cycle arrest through the up-regulation of p21 and p27 in MCF10A-ras cells. Apicidin treatment results in the concentration-dependent activation of ERK1/2, which contributes to apoptosis through the activation of caspase-9 and -6 cleavage. Taken together, these results show that apicidin inhibits the

growth of MCF10A-*ras* and MCF10A cells through cell cycle arrest at the G<sub>1</sub> phase. In MCF10A-*ras* cells, the effect of apicidin appears to be associated with the down-regulation of phosphorylated Rb whose expression appears to be under the control of the cyclin/CDK complex. Cyclin D1 plays an important role in the progression of mammalian cells through the G<sub>1</sub> phase of the cell cycle. The inappropriate overexpression of cyclin D1 is frequently observed in several types of human cancer (25). The CDK inhibitor, p27<sup>Kip1</sup>, also plays an important role in mediating growth arrest and is believed to function as a break in the cell cycle (26,27). p27<sup>Kip1</sup> inhibits the catalytic activities of G<sub>1</sub>-specific cyclin-CDK complexes, such as cyclin E/CDK2, whose activity is essential for the entry of cells into the S phase (5). Another CDK inhibitor, p21<sup>WAF1/Cip1</sup>, which is controlled by a p53-dependent or -independent mechanism at the transcriptional level, is a negative regulator of cell cycle progression (27).

It was previously reported that cyclin D1 is a key mediator of the Ras-induced p21<sup>WAF1/Cip1</sup> stability in *ras*-transformed cells (28). In this study, apicidin reduced the level of cyclin E expression and induced p53 expression in MCF10A-*ras* cells in a concentration-dependent manner, which was accompanied by up-regulation of p21<sup>WAF1/Cip1</sup> and p27<sup>Kip1</sup>. Rb is one of the targets of the cyclin/CDK complexes, which upon hyperphosphorylation dissociate from bound transcription factors, such as E2F, enabling them to activate the genes essential for DNA replication (5). These results demonstrate that apicidin produced a significant decrease in expression of p-Rb levels in MCF10A-*ras* cells. However, the levels of cyclin E and CDK2 proteins were attenuated in apicidin-treated MCF10A cells without altering the p-Rb expression.

The *ras* gene plays an essential role in cellular proliferation and differentiation. Ras phosphorylates and activates Raf and MEK1/2 which, in turn, phosphorylate ERK1/2 (1). In general, activation of the ERK1/2 pathway is normally involved in promoting the survival of most cells, and oncogenic RAS persistently activates the ERK1/2 pathways, which contributes to the increased proliferation of tumor cells (5). However, recent studies have shown that cisplatin and quercetin induce apoptosis in human cancer cells through the activation of ERK (29,30). Pettersson *et al* (31) also suggested that the activation of ERK was essential for the retinoid-induced apoptosis of breast cancer cells. Michaelis *et al* (13) reported that valproic acid increases the level of ERK 1/2 phosphorylation in human umbilical vein endothelial cells. These results provide evidence for a novel signaling pathway that mediates apicidin-induced apoptosis. The level of ERK1/2 phosphorylation was increased and the level of cyclin D1 expression was decreased in MCF10A-*ras* cells treated with apicidin. This suggests that apicidin-induced cell cycle arrest can be achieved by targeting the ERK pathway.

Downstream of the ERK pathway, p53 plays an important role in cell cycle arrest and apoptosis. For example, the gene transfer-mediated increases in the apoptosis-blocking Bcl-2 protein have been reported to interfere with p53-induced apoptosis without impairing p53-induced G<sub>1</sub>/S arrest in tumor cell lines lacking p53 (18). The regulation of p53 expression through the Ras-activated RAF/MEK/ERK pathway has recently been reported in the human fibrosarcoma cell line, HT1080 (32). Once the ERKs are activated by apicidin, the

up-regulation and activation of p53 are the key downstream events, as evidenced in previous reports (32,33). In this study, apicidin induced up-regulation of the p53 protein in MCF10A-*ras* cells (Fig. 5A). Downstream of the p53 pathway, Bax, a direct target of p53, plays an important role in apicidin-induced apoptosis. In MCF10A-*ras* cells, apicidin-induced up-regulation of Bax matches the up-regulation of p53. Bax promotes apoptosis through an interaction with the anti-apoptotic members in the mitochondria, such as Bcl-2 (34). Such interactions have been shown to activate caspases. The morphological and biochemical changes associated with apoptosis are largely caused by activation of a family of intracellular cysteine aspartyl-specific proteases known as caspases, which cleave many vital cellular substrates contributing to cell death and to the apoptotic phenotype (35). Caspase-9 is an initiator caspase in the apoptotic process whose function is to activate effector caspases that occur downstream in the mitochondrial pathway of apoptosis (35). Caspases-6 and -3 are downstream executioners of effector caspases (36,37). In the present study, up-regulation of Bax allowed the activation of caspases-9 and -6 in apicidin-treated MCF10A-*ras* cells. These findings indicate that apicidin exhibits a significant anti-proliferation effect through activation of apoptotic processes that are characterized by the activation of caspases-9 and -6 via a p53-dependent mechanism. However, apicidin decreased levels of p53, Bax, caspase-9 and caspase-6 protein in MCF10A cells. Therefore, apicidin-induced apoptosis in MCF10A cells was shown to be independent of the caspases that are generally accepted as being effectors of the apoptotic process.

We also examined whether ERKs are essential for AP-1 activation. AP-1 transcription factors are involved in the control of cell death and survival. Increased AP-1 activity promotes apoptosis in certain cell types, while promoting survival in others. The ectopic expression of c-Jun or c-Fos can induce apoptosis in mouse fibroblasts, sympathetic neurons and human colorectal carcinoma cell lines (38,39). In this study, apicidin induced the activation of c-Jun but had no effect on the levels of c-Fos protein in both cell lines. This suggests that apicidin might activate the AP-1 transcription factor through an ERK-independent pathway.

These results demonstrate that apicidin inhibits the growth of MCF10A and MCF10A-*ras* cells by increasing the expression of CDK inhibitors (such as p21<sup>WAF1/Cip1</sup> and p27<sup>Kip1</sup>) through a decrease in the level of G<sub>1</sub> cyclin expression, and by decreasing CDK protein levels. In MCF10A-*ras* cells, apicidin leads to down-regulation of Rb hyperphosphorylation and an increase in p53 protein levels. Taken together, apicidin effectively blocks the G<sub>1</sub> phase of the cell cycle, leading to apoptosis. In addition, the specific activation of the ERK pathway markedly increases the susceptibility of MCF10A-*ras* cells to apicidin-induced apoptotic cell death, which appears to be mediated by the activation of caspases through a p53-dependent mechanism. Such caspase-dependent cell death was induced only in MCF10A-*ras* cells with the constitutive activation of ERK. This shows that apicidin-induced activation of the ERK pathway in H-*ras*-transformed cells induces anti-proliferative activity through the cell cycle arrest at the G<sub>1</sub> phase and increases the caspase-dependent apoptotic potential (Fig. 6). Therefore, targeting the activated



ERK pathway in *ras*-transformed breast cells may help increase the effectiveness of many chemotherapeutic strategies in the treatment of *ras*-related breast cancer.

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