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Apigenin Regulates Matrix Metalloproteinase-2/9 and Rho GTPase Family through FAK Signal to Reduce Breast Cancer MCF-7 Cells Metastasis

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Abstract

Cancer metastasis is the toughest problem in cancer therapy. Once tumor cells have the ability of surrounding cell invasion, the survival rate of patient with tumor disease will be reduced. Hence, prevent or reduce the cancer metastasis will can increase the patients' survival rate. Apigenin has been demonstrated optimal effect in cancer prevention and reduce cancer metastasis, including breast cancer. In this study, we verify low dose (≤ 5 $\mu\text{g/ml}$) apigenin exposure 3 h would not influence the MCF-7 cells viability but can decrease its FAK signal activation to reduce matrix metalloproteinases (MMP-2 and MMP-9) expression. Moreover, low dose apigenin treated in MCF-7 cells could reduce the cell mobility through the Rho GTPases (Ras, Rac-1, cdc-42, and RhoA) downregulation to cause cytoskeleton remodeling. Presented results have demonstrated the role of apigenin on breast cancer metastasis is associated with FAK signal pathway inhibition. The capacity of low dose apigenin treated in breast cancer for short time has been clarified here. And thus may find widespread application in clinical therapy as an anti-metastatic medicament.

Keywords: Cancer Metastasis, Apigenin, Matrix Metalloproteinases, Rho GTPases.

Introduction

Breast cancer is the common cancer of women and the ranking 5th leading cause of death worldwide [1]. The risk factor of breast cancer development including, obesity, drinking alcohol, early age at first menstruation, having children late or not at all, older age, etc. About 5–10% of cases are genomic alteration, including BRCA1 and BRCA2 mutation [2]. Surgery is the major method to remove the tumor. Chemotherapy, radiation therapy and hormone therapy are often used to reduce the recurrence. The majority of early-stage breast cancer patients was undergo breast conserving surgery and then receive adjuvant treatment. The 5-years survival of early-stage breast cancer is 98.6%. While in late-stage, often associated with metastasis, these therapies could not achieve the optimal treatment and cause the survival rate declines to 24.3% [3]. Furthermore, once breast cancer with metastasis will cause poor outcome. Therefore, inhibition of breast cancer metastasis can extend the patients' survival.

Cancer metastasis is a complex process, the local tumor cells

migrate to distant site by the bloodstream, the lymphatic system, or by direct extension. Among them, the epithelial-mesenchymal transition (EMT) is the important characteristics. EMT is the conserved developmental process in the evolution [4], and it also plays pivotal role in cancer metastasis. Cytokines and growth factors, such as transforming growth factor β (TGF- β) are related with EMT dysregulation during the process of malignant tumor [5]. In the EMT process, the cell-cell junction would be breakdown by E-cadherin reducing, and the Rho GTPase function also be modulated, loss of cell polarity ensues, a dramatic reorganization of the actin cytoskeleton to enhance cell motile characteristics [5-7]. Once cancer metastasis, the survival rate of cancer patients would be reduced. But, the molecular mechanism of cancer metastasis happen is unclear. Therefore, in clinical, inhibition of cancer metastasis is the crucial issues to prolong the life of tumor diseases patients.

Apigenin (4',5,7-trihydroxyflavone) is a plants source natural product, belonging to the flavone class. The functions of apigenin

are recognized as anti-inflammatory, antioxidant and anticancer. Apigenin has been shown effective in inhibiting several cancers, including breast cancer [8], lung cancer [9], oral cancer [10], gastric cancer [11], colon cancer [12], bladder cancer [13], prostate cancer [14]. It also can induce autophagy in leukemia cell, and simultaneously induces resistance against the vincristine [15]. Moreover, apigenin treatment can enhance cell apoptosis through down regulate bcl-2 expression [16]. Epidemiologic studies indicate that flavones can decrease cancers development, particularly breast cancer [17]. In breast cancer, apigenin has been demonstrated can induce cell apoptosis by inhibiting STAT3 and NFκB signaling [18] and inhibit metastasis through blocking PI3K/Akt pathway and β4 integrin function [19]. These literatures imply apigenin may be involved in EMT change to inhibit breast cancer metastasis. Thus, we try to clarify the inhibition mechanism of breast cancer metastasis through apigenin treatment in this study.

Materials and methods

Reagents and antibodies

Apigenin (Purity > 99%), was purchased from Extrasynthese (Genay, France); DMSO, Tris- HCl, EDTA, SDS, bovine serum albumin (BSA), gelatin, crystal violet, and sodium orthovanadate were purchased from Sigma-Aldrich (St. Louis, MO, USA); the protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Dulbecco's phosphate buffer solution (PBS), trypsin-EDTA, Dulbecco's modified Eagle's medium (DMEM) purchased from Life Technologies, Inc. (Gibco/BRL, Gaithersburg, MD). Antibody against FAK, MAPK, and PI3K/Akt, proteins and phosphorylated proteins were purchased from Cell Signaling Tech. (Beverly, MA, USA). PI3K, MMP-2, MMP-9, Ras, Rac-1, Cdc42, RhoA, RhoB, NF-κB, c-Fos, c-Jun, β-actin, and C23 antibodies were purchased from BD Transduction Laboratories (San Jose, CA, USA). The enhanced chemiluminescence (ECL) kit was purchased from Amersham GE Healthcare UK Ltd. (Buckinghamshire, England).

Cell culture and apigenin treatment

Human breast cancer cells, including MCF-7, MDA-MB-231, and H184B5F5/M10 were maintained in DMEM medium containing with 10% fetal calf serum and antibiotics (100 U/ml of penicillin and 100 mg/ml of streptomycin). All cells were cultured at 37°C in a humidified atmosphere of 5% CO₂-95% air. The stock solution of apigenin was dissolved in dimethylsulfoxide (DMSO) and sterilized by filtration through 0.2 μm disc filters. Suitable amounts of stock solution (10 mg/ml in DMSO) of apigenin were added into the cultured medium to achieve the indicated concentrations (final DMSO concentration was < 0.2%).

Assessment of cell viability (MTT assay)

To explore the effect of apigenin on breast cancer cell viability, the various concentration (0, 1, 2.5, 5, 7.5, 15, 30, and 45 μg/ml) were respectively treated in MCF-7, MDA-MB-231, and H184B5F5/M10 cell lines for 24 and 48 h. At the end of the assay period, cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay. Each concentration was repeated three times. After the exposure period, the medium was

removed and followed by washing of the cells with PBS. Then, the medium was changed and incubated with MTT solution (5 mg/ml) per well for 4 h. The medium was removed, and formazan was solubilized in isopropanol and measured spectrophotometrically at 563 nm. The percentage of viable cells was estimated by comparing with the untreated control cells (0 μg/ml).

Boyden chamber invasion and migration assay

Matrigel (BD Transduction Laboratories, San Jose, CA) was diluted to 200 μg/ml with cold filtered distilled water, and then added to the upper chamber with 8μm pore polycarbonate filter. And then the treated MCF-7 cells (1×10⁵ cells/well) were mixture with serum free medium to add into the upper chamber. Culture medium containing with 10% fetal bovine serum was applied to the lower chamber as chemoattractant. The chamber was incubated for 8h at 37°C. At the end of incubation, the cells in the upper surface of the membrane were carefully removed with a cotton swab. The invasive cells were fixed by 3% methanol and then stained by 5% Giemsa solution. The invading cells on the lower surface of the membrane filter were counted with a light microscope. The data are presented as the average number of cells attached to the bottom surface from five random fields. Each experiment was carried out in triplicate.

To measure the migrative ability of MCF-7 cells, cells were seeded on the upper surface of the filter inserts with 8-μm pore polycarbonate filters that were not coated with matrigel. The migrative cells were treated and measured as described in the invasion assay.

Wound-healing assay

To determine cell motility determination, MCF-7 cells (1×10⁵cells/ml) were seeded in 6-well tissue culture plate and grown to 80-90% confluence. After aspirating the medium, the center of the cell monolayers was scraped with a sterile micropipette tip to create a denuded zone (gap) of constant width. Subsequently, cellular debris was washed with PBS, and the cells were exposed to various concentrations of apigenin (0, 1, 2.5, and 5 μg/ml). The wound closure was monitored and photographed at 0, 6, 12, 24, and 48 h with an Olympus CKX-41 inverted microscope and an Olympus E-410 camera. To quantify the migrated cells, pictures of the initial wounded monolayers were compared with the corresponding pictures of cells at the end incubation. Artificial lines fitting the cutting edges were drawn on pictures of the original wounds and overlaid on the pictures of cultures after incubation. Migrated cells across the white lines were counted in six random fields from each triplicate treatment, and data are presented as mean ± SD.

Immunofluorescence assay

To determine the effect of apigenin on EMT and cytoskeleton change, MCF-7 cells (4×10⁵ cells/well) were plated in six-well plates and grown for 24 h and then incubated in the different concentrations of apigenin (0, 1, 2.5, and 5 μg/ml) for 24 h. After the exposure period, the cells were washed with Ca²⁺/Mg²⁺ free PBS after cell medium removed. Cells were fixed with 4% paraformaldehyde in PBS for 15 min and incubated with 0.5% Triton X-100 in PBS for 5 min. Cells were incubated with 1%

bovine serum albumin in 0.5% PBST at room temperature for 1 h. And then add the primary antibody or phalloidin-PE (Invitrogen, Karlsruhe, Germany) to incubate at room temperature for 1 h. The fluorescent images were visualized with a BX51 fluorescence microscope (Olympus, Tokyo, Japan).

Analysis of MMP-2/9 activity by gelatin zymography

MCF-7 cells (4×10^5 cells/ml) were seeded into the 10 cm culture dish and then incubated in the different concentrations of apigenin (0, 1, 2.5, and 5 $\mu\text{g/ml}$) for 24 h. Subsequently, the conditioned medium was collected and gelatin zymography was performed to examine the activities of MMP-2 and MMP-9. Samples were mixed with loading buffer and electrophoresed on 8% SDS-polyacrylamide gel containing 0.1% gelatin. Electrophoresis was performed at 140 and 110 V for 3 h. Gels were then washed twice in zymography washing buffer (2.5% Triton X-100 in double-distilled H₂O) at room temperature to remove SDS, followed by incubation at 37°C for 12-16 h in zymography reaction buffer (40 mM Tris-HCl, 10 mM CaCl₂, 0.02% NaN₃), stained with Coomassie blue R-250 (0.125% Coomassie blue R-250, 0.1% amino black, 50% methanol, 10% acetic acid) for 1 h and destained with destaining solution (20% methanol, 10% acetic acid, 70% double-distilled H₂O). Nonstaining bands representing the levels of the latent forms of MMP-2 and MMP-9 were quantified by densitometer measurement using a digital imaging analysis system.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from MCF-7 cells using the total RNA Extraction Midiprep System (Viogene BioTek, Taiwan). Total RNA (2 μg) was transcribed to 20 μl cDNA with 1 μl deoxynucleotide triphosphate (dNTP; dNTP set consists of 2.5 mM aqueous solutions at pH 7.0 of each of dATP, dCTP, dGTP, and dTTP), 1 μl Oligo dT (10 pmol/ml), 1 μl RTase (200 U), 1 μl RNase inhibitor and 5 \times reaction buffer. The appropriate primers (sense of MMP-2, 5'-GGCCCTGTCCTCCTGAGAT -3', nt 1337-1356; antisense of MMP-2, 5'-GGCATCC AGGTTATCGGGGA-3', nt 2026-2007; sense of MMP-9, 5'-AGGCCTCTACAGAGTCTT TG-3', nt 1201-1220; antisense of MMP-9 were used for polymerase chain reaction (PCR) amplifications. PCR was performed with Platinum Taq polymerase (Invitrogen, San Diego, CA, USA) under the following conditions: 30 cycles of 94°C for 1 min, 59°C (MMP-2) or 60°C for 1 min (MMP-9 and β -actin), 72°C for 1 min followed by 10 min at 72°C. The final products were electrophoresis in 2% agarose gel and then detected through ethidium bromide staining.

Preparation of whole-cell lysates and nuclear extracts

Lysis cell in iced-cold RIPA buffer (1 % NP-40, 50 mM Tris-base, 0.1% SDS, 0.5% deoxycholic acid, 150 mM NaCl, pH 7.5) and then add the protease inhibitors (phenyl- methylsulfonyl fluoride (100 mM), leupeptin (100 mM)), and sodium orthovanadate (100 mM). After vortexing for 30 min on ice, the samples were centrifuged at 12000 \times g for 10 min, and then the supernatants were collected, denatured and subjected to SDS-PAGE and Western blotting. Nuclear extracts were prepared as previously described. Each nuclear pellet was resuspended in nuclear extract buffer (1.5 mM MgCl₂, 10 mM HEPES, pH 7.9, 0.1 mM EDTA,

0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol, and 420 mM NaCl). The nuclear suspension was incubated on ice for 20 min and then centrifuged at 14,000 \times g for 5 min. The supernatant (corresponding to the soluble nuclear fraction) was saved, and then used for NF- κ B (p65 and p50), c-Jun and c-Fos detection. The protein content was determined with Bio-Rad protein assay reagent using bovine serum albumin as a standard.

Western blotting analysis

The denatured samples (50 μg extracted protein) were resolved on 10-12% SDS-PAGE gels. The proteins were then transferred onto nitrocellulose membranes. Non-specific binding of the membranes was blocked with Tris-buffered saline (TBS) containing 1% (w/v) nonfat dry milk and 0.1% (v/v) Tween-20 (TBST) for more than 2 h. Membranes were washed with TBST three times for 10 min and incubated with an appropriate dilution of specific primary antibodies in TBST overnight at 4°C. Subsequently, membranes were washed with TBST and incubated with appropriate secondary antibodies (horseradish peroxidase-conjugated goat antimouse or antirabbit IgG) for 1 h. After washing the membranes three times for 10 min in TBST, the bands detection were revealed by enhanced chemiluminescence using ECL Western blotting detection reagents and exposed ECL hyperfilm in FUJIFILM Las-3000 mini (Tokyo, Japan). Then proteins were quantitatively determined by densitometry using FUJIFILM-Multi Gauge V3.0 software.

Statistical analysis

Data were expressed as means \pm standard deviation of three independent experiments and statistical analysis was obtained using a Student's t-test. All statistical analyses of data were performed using Sigma Plot 2001 software (Systat Software Inc., San Jose, Calif., U.S.A.). Significant differences were established at $P \leq 0.05$.

Results

Low dose ($\leq 5 \mu\text{g/ml}$) apigenin no effect the cell viability, but suppress the MCF-7 cells migration and invasion

To explore the optimal concentration of apigenin to influence breast cancer cell, the MTT assay is used here. The concentration of apigenin including 0, 1, 2.5, 5, 7.5, 15, 30, and 45 $\mu\text{g/ml}$ were respectively treated in MCF-7, MDA-MB-231 and H184B5F5/M10 breast cancer cell lines. After treatment for 24 or 48 h, we noticed low dose ($\leq 5 \mu\text{g/ml}$) apigenin no toxicity in MCF-7 cells, and the cell viability can achieve 80%. However, once the dose of apigenin more than 5 $\mu\text{g/ml}$, the cell viability dramatic decrease. But, this phenomenon could not observe in MDA-MB-231 cells (Fig. 1a). In pathologic characteristics, the MDA-MB-231 cell is basal type breast cancer cell and associated with poor prognosis, in turn, MCF-7 cell is a luminal type breast cancer have better 5-years survival rate. Imply the low dose apigenin treatment in luminal type breast cancer can achieve the optimal treat efficiency more than in basal type.

Next, we explore the role of apigenin in MCF-7 cells under the low dose treatment. Apigenin has been shown can inhibit invasion and

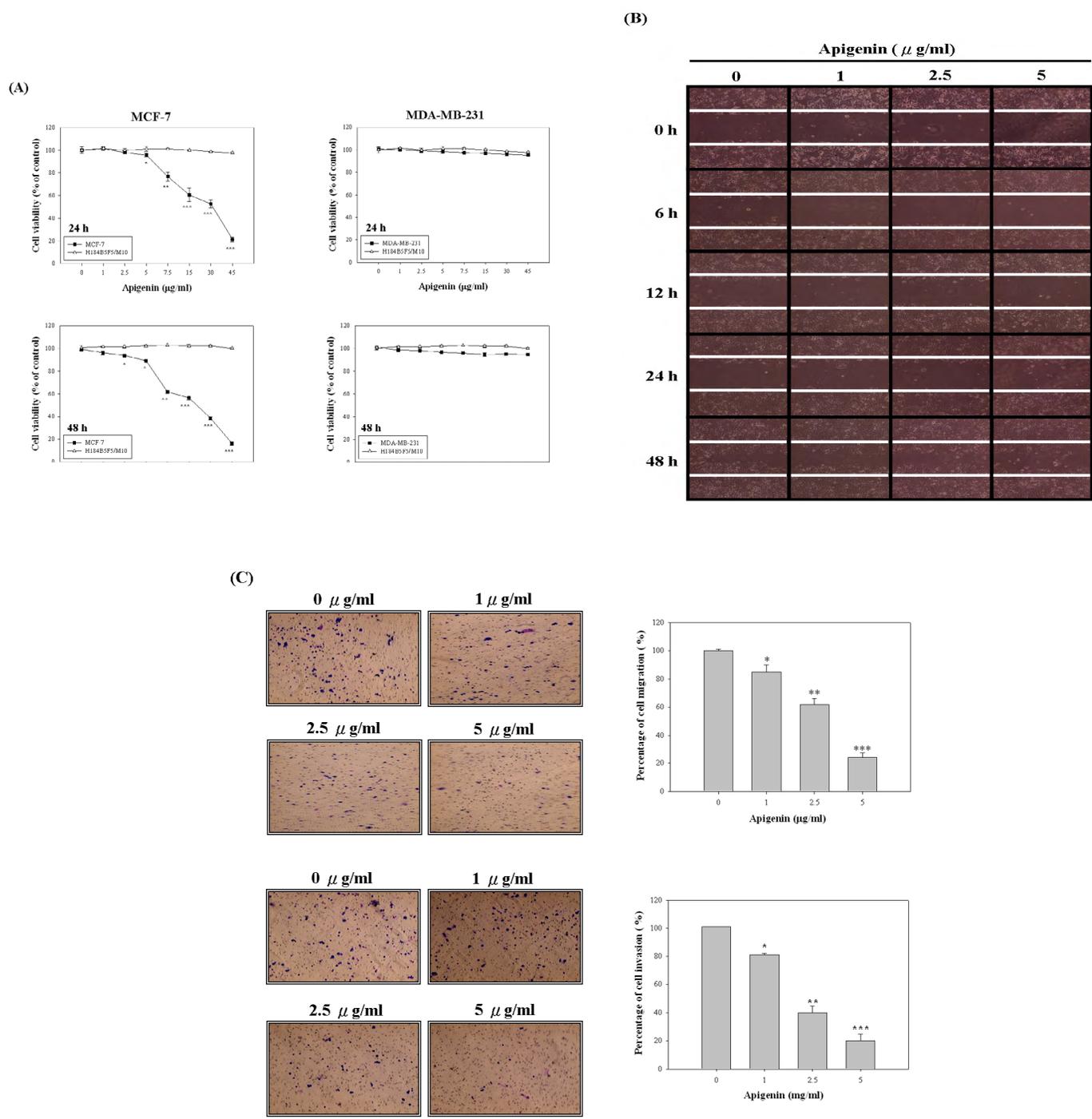


Fig. 1 Low dose apigenin treatment reduces cell migration and invasion. (a) MTT assay to determine cell viability of MCF-7 and MDA-MB-231 cells, and found the low dose (0-5 $\mu\text{g/ml}$) apigenin no effect cell viability in MCF-7 cells as compared with normal cell line, H184B5F5/M10. It is worth mentioning that MDA-MB-231 cells viability would not decrease in the apigenin presence. That is to say, low dose apigenin treatment achieves optimal effect is manifested in MCF-7 cells rather than MDA-MB-231 cells. (b) The time-dependant manner of MCF-7 cells migration can be inhibited by the low dose apigenin exposure 6, 12, 24 or 48 h to enter the tip scratch area. (c) The dose-dependant manner of MCF-7 cells invasion significantly reduced by the apigenin exposure 24 or 48 h. *P as $p<0.05$; **P as $p<0.01$; ***P as $p<0.001$.

migration in colorectal cancer^[20]. In cell migration assay and cell invasion assay, we observe the phenomenon of cell migration was reduced and the invasive cells were significantly decreased (Fig. 1b and Fig. 1c). These results suggest that the optimal concentration of apigenin in breast cancer cell treatment is $\leq 5 \mu\text{g/ml}$ to reduce cell migration and invasion.

Low dose apigenin reduce the MMP-2/9 and EMT expressions

Known the low dose apigenin can reduce the MCF-7 cells migration and invasion during 24 h treatment, we next to verify the MMP-2 and MMP-9 expression under 3 h exposure of low dose apigenin. In the zymography results, we observed the MMP-2 and MMP-9 decreasing in MCF-7 cells after low dose apigenin exposure (Fig. 2a). In the meantime, we noticed the RNA level of MMP-2 and MMP-9 were not change (Fig. 2b), suggest that exposure 3h of low dose apigenin is influence protein expression rather than genomic change. The results imply the apigenin can inhibit breast cancer metastasis through matrix metalloproteinase and plasminogen activator down regulation.

We next try to clarify the EMT change whether manifested in MCF-7 cells after exposure 3 h of low dose apigenin. After fluorescence

microscopy imaging, we noticed the E-cadherin increase and the fibronectin decrease in MCF-7 cells under the low dose apigenin exposure (Fig. 2c). These results imply the low dose apigenin can inhibit MCF-7 cells metastasis through MMP-2 and MMP-9 down regulation. In addition, it also can down-regulate EMT after apigenin exposure 3 h.

Apigenin inhibit the MCF-7 cells metastasis through FAK signals

To clarify the molecule mechanism of breast cancer metastasis could be inhibited by low dose apigenin exposure 3 h, we use the western blot experiment to demonstrate. Literatures indicate apigenin can regulate PI3K/Akt signal pathway to inhibit breast cancer cell metastasis^[19], and promote apoptosis via a caspase-dependent mechanism^[21]. The PI3K/Akt signal is the down stream of Focal Adhesion Kinase (FAK). FAK transmits signal to the cell interior through the candidate proteins phosphorylation and dephosphorylation to cause MMP-9 decreased while metastasis reduced^[22]. However, we noticed the MMP-2, and MMP-9 were decreased after low dose apigenin exposure for 3 h, imply FAK signal is the major pathway to regulate breast cancer cell

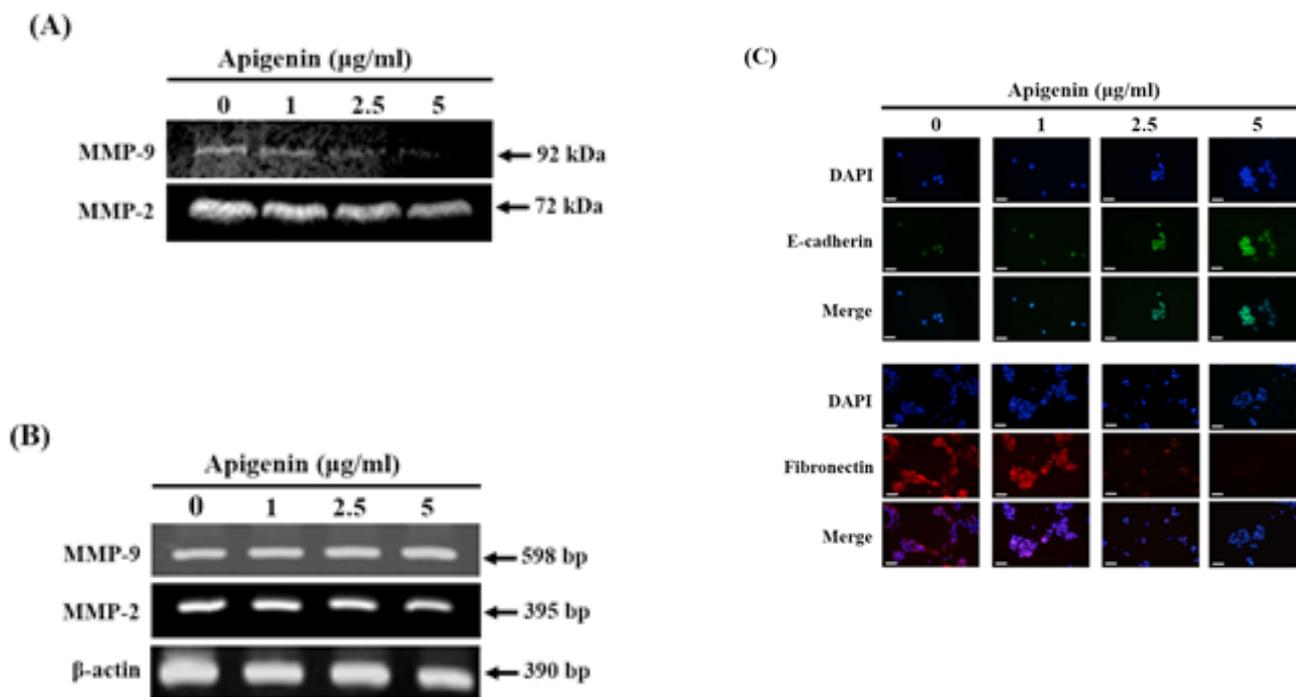


Fig. 2 Low dose apigenin can influence the matrix metalloproteinase expression and EMT. (a) Galetin zymography assay was used to determine the MMP-2 and MMP-9 expression under low dose apigenin exposure 3 h. We noticed the MMP-2 and MMP-9 were be decreased by low dose apigenin especially in the concentration of 5 $\mu\text{g/ml}$. (b) RT-PCR data indicated that the MMP-2 and MMP-9 expression decrease in low dose apigenin exposure 3 h is not involved in genomic change. (c) Fluorescence images show that the dose-dependent manner of MCF-7 cells treated with low dose apigenin could cause E-cadherin slight increase and fibronectin decrease. Scale=50 μm .

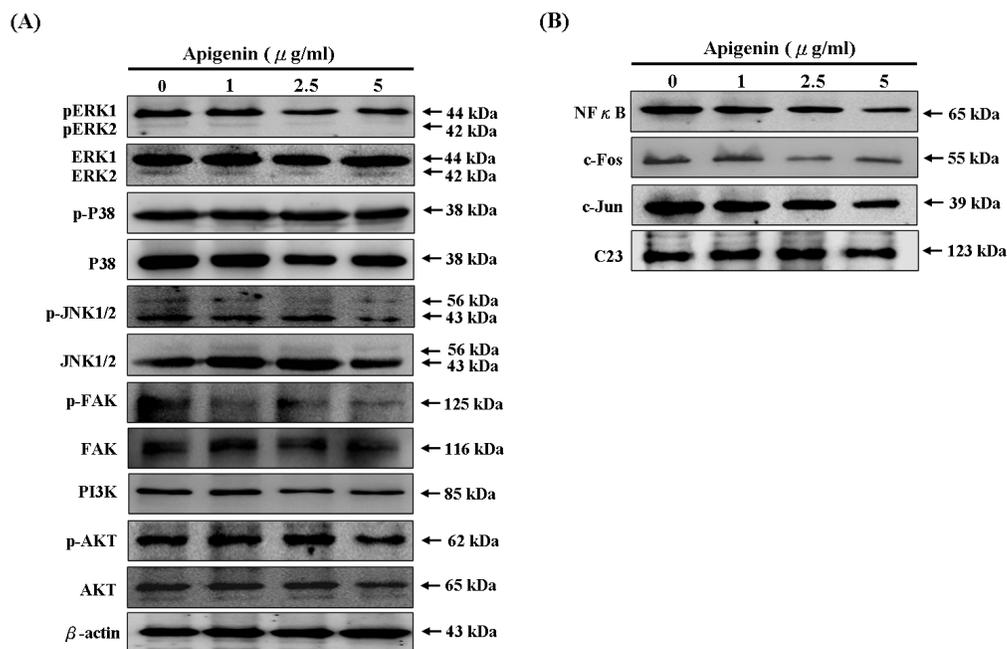


Fig. 3 Low dose apigenin can trigger FAK deactivation to inhibit metastasis. (a) After low dose apigenin exposure 3 h, the FAK/JNK signal was observed downregulation in MCF-7 cells. ERK and PI3K/Akt signals were not observed change in the low dose apigenin exposure 3 h. (b) The nuclear protein, including NFκB, c-Fos, and c-Jun were also be observed downregulation in MCF-7 cells after low dose apigenin exposure 3 h.

also slightly decreased. In the meantime, the nuclear NFκB, c-Fos, and c-Jun were downregulated (Fig. 3b), illustrate the FAK signal can be inhibited by apigenin exposure 3 h.

Apigenin can regulate the cytoskeleton change to inhibit the MCF-7 cells metastasis

As described above, low dose apigenin exposure 3 h down-regulate MMP-2 and MMP-9 through FAK inhibition. On the other hand, we also noticed cytoskeleton regulator influence in the low dose apigenin exposure 3 h. The Ras, Rac-1, Cdc42, and RhoA were the downstream of FAK associated with cytoskeleton remodeling.

Known the Ras and Rac-1 are related with lamellipodia, the Cdc42 is a regulator in filopodia, and the RhoA is associated with stress fiber formation. Here, we observed the Ras and Rac-1 obviously down regulation after low dose apigenin exposure 3 h as compared with Cdc-42 and RhoA (Fig. 4a). After fluorescence microscopy imaging, we also observed the lamellipodia was not obviously express in MCF-7 cells after low dose apigenin exposure 3 h (Fig. 4b). The results suggest that apigenin inhibit breast cancer cell metastasis is through FAK signal down regulation to cause matrix metalloproteinase decreasing and cytoskeleton remodeling (Fig. 5).

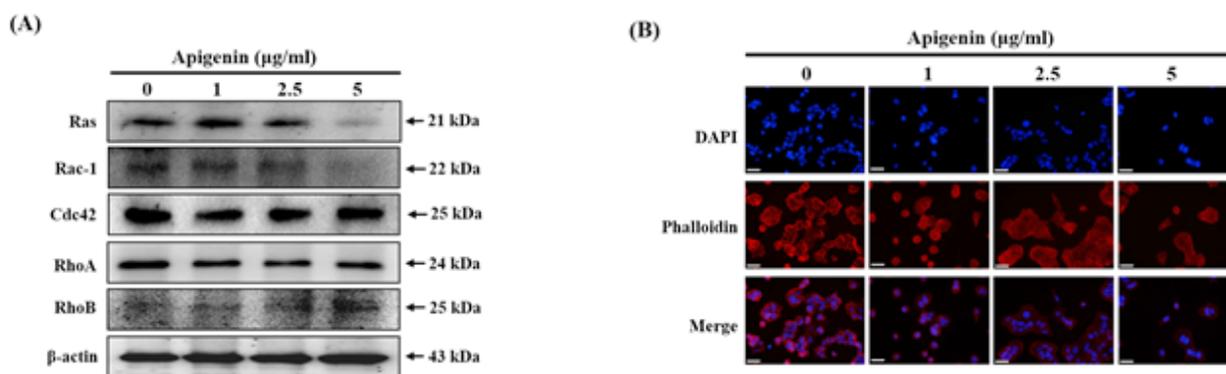


Fig. 4 Low dose apigenin can influence the cytoskeleton. (a) The cytoskeleton regulator, including Ras, Rac-1, Cdc42, RhoA, and RhoB were determined by western blot assay. The Ras and Rac-1 were markedly decreased, and the Cdc42 and RhoA were slight decreased in MCF-7 cells after low dose apigenin exposure 3 h. (b) Phalloidin-PE stain in MCF-7 cells after low dose apigenin exposure 3 h, and then microscopy imaging to explore the cytoskeleton change. The cytoskeleton remodeling was clearly observed, like the lamellipodia reduce in MCF-7 cells after the low dose apigenin exposure 3 h. Scale=50 μm.

metastasis in this study. Thus, we try to analysis the ERK1/2, p38, JNK1/2, and PI3K that are the FAK signals candidates, and noticed the JNK signal was dramatically inhibited in the MCF-7 cells after apigenin treatment (Fig. 3a). The ERK1/2 and Akt were also slightly decreased. In the meantime, the nuclear NFκB, c-Fos, and c-Jun were downregulated (Fig. 3b), illustrate the FAK signal can be inhibited by apigenin exposure 3 h.

Apigenin can regulate the cytoskeleton change to inhibit the MCF-7 cells metastasis

As described above, low dose apigenin exposure 3 h down-regulate MMP-2 and MMP-9 through FAK inhibition. On the other hand, we also noticed cytoskeleton regulator influence in the low dose apigenin exposure 3 h. The Ras, Rac-1, Cdc42, and RhoA were the downstream of FAK associated with cytoskeleton remodeling. Known the Ras and Rac-1 are related with lamellipodia, the Cdc42 is a regulator in filopodia, and the RhoA is associated with stress fiber formation. Here, we observed the Ras and Rac-1 obviously down regulation after low dose apigenin exposure 3 h as compared with Cdc-42 and RhoA (Fig. 4a). After fluorescence microscopy imaging, we also observed the lamellipodia was not obviously express in MCF-7 cells after low dose apigenin exposure 3 h (Fig.

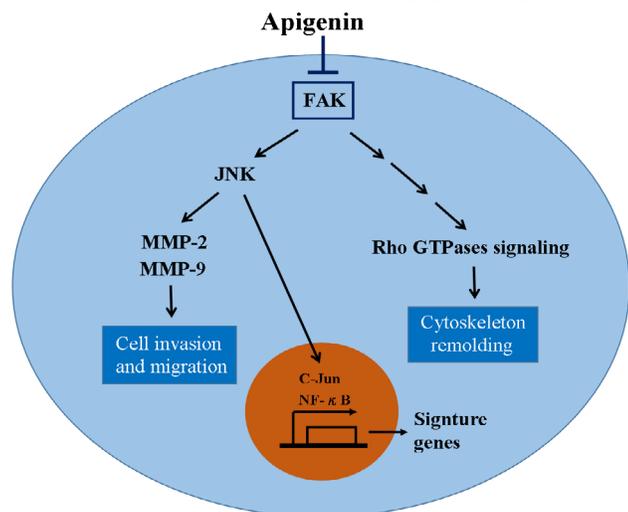


Fig. 5 The cartoon drawing is summarizing the effect of low dose apigenin exposure 3 h in MCF-7 cells. Low dose apigenin can trigger FAK deactivation in MCF-7 cells to reduce cell migration and invasion through JNK signal downregulation. On the other hand, FAK deactivation also can influence the cytoskeleton remodeling through the cytoskeleton regulator inhibition.

4b). The results suggest that apigenin inhibit breast cancer cell metastasis is through FAK signal down regulation to cause matrix metalloproteinase decreasing and cytoskeleton remodeling (Fig. 5).

Discussion

Tumor metastasis is the toughest problem in cancer therapy. Once tumor cell metastasis, anticancer drugs could not achieve the optimal efficiency, and the survival rate of patients with cancer also

would reduced. Thus, avoid or reduce the phenomena of cancer metastasis is the most important issue in the cancer research. Cancer metastasis is the process of EMT. The epithelial cells lose their cell polarity and cell-cell adhesion, and enhance its migratory and invasive properties to become mesenchymal cell type. EMT is also critical for development of many tissues and organs in the developing embryo and numerous embryonic events [23]. Many markers have been considered to be a fundamental event in EMT, such as loss expression of E-cadherin, type IV collagen or laminin

In the meantime, increase the expression of N-cadherin, vimentin, or fibronectin...ect [24]. EMT is also involved in cytoskeleton remodeling [25] and the extracellular matrix (ECM) damaged [26]. Matrix metalloproteinases (MMPs) play key functions in degrade and modify the ECM, gelatin-cleaving MMPs (MMP-2 and -9) were the frequently molecule explored in cancer research with metastasis. In this study, we verify the MMP-2 and -9 were down regulation after low dose apigenin exposure 3h.

On the other hand, EMT is also a process of cytoskeleton change. The actin cytoskeleton is a highly dynamic structure manifested in all live cells. It is based on well-balanced of the local assembly and disassembly of actin filaments. Rho GTPases are the mainly responsible of transmitting signals from chemokine and growth factor receptors and from adhesion receptors to the effector of actin remodeling candidates, including RhoA, Rac1 and Cdc42. The activity of RhoA during EMT is effects cell-cell adhesion, Ras and Rac1 enhance the formation of protrusive membrane structures, such as lamellipodia and Cdc42 can influence filopodia that are rod-like extension consisting of tight bundled actin fibers which penetrate into the surrounding environment originating from the basis of lamellipodia. In this study, we noticed the Ras and Rac1 is decreased after low dose apigenin treatment implies the low dose apigenin can decrease lamellipodia formation to reduce cell motility.

Apigenin has been demonstrated strong inhibited HER2/neu over-expressing breast cancer cells growth, but it was much less effective in inhibiting growth of cells expressing basal levels of HER2/neu [27]. Many studies were verifying the effect of apigenin on breast cancer cell (Tab. 1). In these studies, 24-48 h exposure time of apigenin treated in MCF-7 or MDA-MB-231 cells were the most used to against the cell growth. The MAPK and PI3K/Akt pathways were the most clarify in these studies. However, FAK signal is the less described in breast cancer cell after apigenin exposure. Here, we observed the cell growth could not be inhibited in basal type breast cancer cell line, MDA-MB-231 in the 1-45 μg/ml of apigenin exposure 24 or 48 h. Suggest that apigenin treated in basal type breast cancer cell needs to take longer exposure time. That is to say, low dose apigenin exposure 3 h in breast cancer can achieve optimal effect is in the luminal type rather than basal type. Nevertheless, low dose apigenin treatment is sufficient to effect breast cancer cell cytoskeleton remodeling and decreasing the matrix metalloproteinases expression through FAK signal to reduce metastasis were verified in this study. In terms of cancer therapy, low dose apigenin exposure short time may be the potential for reduces lumina type cancer cell metastasis.

Tab. 1 The study of breast cancer and apigenin effect were described in below.

Year	Author	Cell line	Apigenin conc.	Incubation time	Target molecule	Inhibition
2001	Yin F, et al	MCF-7 and MDA-MB-468	7.8 or 8.9 µg/ml	72 h	cyclin-CDK regulators, ERK MAP kinase	Cell cycle
2001	Lindenmeyer F, et al	MDA-MB-231	2.5 µg/ml (22.8µM)	24 and 48h	MAPK	Protease expression, cell migration, cell adhesion to the ECM, and cell proliferation
2004	Way TD, et al	MDA-MB-453, BT-474, and SKBr-3	40 µM	24 and 48h	PI3K/Akt	Cell growth (Induce apoptosis by depleting HER2/neu protein)
2006	Seo HS, et al	MCF-7 and T47D cells (ERα positive) MDA-MB-435 (ERα negative)	0.1% of culture medium			Cell proliferation
2006	Mak P, et al	MDA-MB-231	20 µM	24h	ERβ	Cell growth
2006	Fang J, et al	MCF-7	20 µM	1h	HIF-1α and VEGF	Angiogenesis
2007	Chen D, et al	MDA-MB-231	25–100 µM	24h	Chymotrypsin-like proteasome	Chymotrypsin-like proteasome activity
2008	Long X, et al	MCF-7	10 µM	24h	p38, PKA, MAPK and AKT	Antiestrogen-resistant breast cancer cell growth
2008	Lee WJ, et al	MDA-MB-231	40 µM	12 and 24h	PI3K/Akt and integrin β4 function	HGF-induced invasive growth
2009	Choi EJ, et al	SK-BR-3	1–100 µM	24 and 48h	CDK1, p21 ^{Cip1} and p53	Cell cycle
2010	Mafuvadze B, et al	T47-D and BT-474	50 or 100 µM		VEGF	Angiogenesis
2013	Cao X, et al	T47-D and MDA-MB-231	6 µM	24 and 48h	PI3K/Akt/mTOR	cell proliferation (through inducing apoptosis and autophagy)
2014	Harrison ME, et al	MDA-MB-231, MBA-MB-468, MCF-7, SK-BR-3	10–30 µM	24 and 72h	PI3K/Akt	Cell growth, oxidative stress, and hypophosphorylation of Akt
2014	Bai H, et al	MCF-7	80 µM		ROS	Cell growth
2014	Seo HS, et al	MDA-MB-231	0–60 µM	72h	STAT3	Cell proliferation

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Abbreviations

MMPs	Matrix metalloproteinases
ERK	Extracellular signal-regulated kinase
PI3K	Phosphoinositide 3-kinase
FAK	Focal adhesion kinase
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
ECL	Enhanced-chemiluminescent
NF- κ B	Nuclear factor kappa B

Reference

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