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Galangin Inhibits Tumor Growth and Metastasis of B16F10 Melanoma

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ABSTRACT

Galangin, an active flavonoid extracted from the root of the *Alpinia officinarum Hance*, showed a cytotoxic effect on several cancer cell lines in vitro. However, there is no information available concerning its antimetastatic effect. Focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase, is involved in many aspects of cellular processes such as proliferation, adhesion, and invasion. Studies have shown that FAK is a promising target for therapeutic intervention in melanoma. In the present study, proliferation of B16F10 cells was suppressed when exposed to various doses of galangin. Inhibition on proliferation by galangin was also detected by clonogenic survival assay. The capabilities of cell adhesion, cell spreading, and cell motility were impaired by galangin, reinforced by F-actin rearrangement. Molecular data showed that both FAK mRNA level and protein level were reduced dose-dependently. Additionally, galangin reduced phosphorylation of FAK (Tyr397) protein. Transient transfection reporter assays showed that galangin suppressed the transcription of FAK gene, indicating FAK expression is a candidate target of galangin. The antimetastatic function of galangin is further supported by the fact that it could inhibit the formation of tumor colonies in the lung tissue on C57BL/6J mouse lung metastatic model using B16F10 melanoma cells. Immunochemical analyses showed that galangin decreased FAK expression in vivo. These data add to our new understanding that galangin can inhibit B16F10 melanoma metastasis both in vivo and in vitro, and that FAK is a valid therapeutic target against melanoma. J. Cell. Biochem. 114: 152–161, 2013.

KEY WORDS: MALIGNANT MELANOMA; GALANGIN; METASTASIS; FAK

Melanoma is the most serious type of skin cancer in the world, accounting for about 80% of deaths of all skin cancer [Shore et al., 2011]. The major health threat for malignant melanoma is death of cancer metastasis. Clinical trials indicated that melanoma shows preferential metastasis to the lung, brain, liver, and skin; meanwhile, it is highly resistant to conventional chemotherapy [Cummins et al., 2006]. The most patients develop metastasis with the 5-year survival rate being only 14% [Chen et al., 2010; Boyle, 2011]. Given the rising incidence of melanoma and the lack of effective therapies, looking for new chemicals that targeting the complex genetic networks involved in melanoma metastasis offers a new avenue for this devastating disease [Pisano et al., 2007; Hocker et al., 2008].

Focal adhesion kinase (FAK), a 125 kDa nonreceptor protein tyrosine kinase, was first isolated from chicken and mouse fibroblasts and then from human T and B lymphocytes and human

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sarcoma samples. Structurally, Human FAK protein shares 95–97% amino acid identity with mouse and chicken [Whitney et al., 1993]. It is activated by diverse signaling molecules that mediates cell spreading, differentiation, proliferation, apoptosis, migration, and invasion [Golubovskaya, 2010; Hall et al., 2011]. FAK is identified as a key mediator implicated in aggressive melanoma phenotype. In malignant melanomas, FAK gene amplification, as well as protein overexpression, correlates well with increased invasion and migration [Chatzizacharias et al., 2007]. Stably expressing FAK related non-kinase (FRNK) resulted in a decrease in FAK phosphorylation. The migration capability was decreased 90% of the control in the K1735 mouse model [Li et al., 2001]. Li et al. [2007] showed that both of targeting FAK via RNAi against FAK or expressing dominant negative FRNK in B16F10 tumor cells and tissues effectively reduced tumor progression and metastasis. Compounds which decrease the expression or/and activity of FAK could be a potential candidate for therapeutic intervention in the treatment of cancer [Schultze and Fiedler, 2010].

Over the past years, there is a growing interest in phytochemicals with anticancer potential and low toxicity [Parekh et al., 2009; Qi et al., 2010]. Galangin (3,5,7-trihydroxyflavone; Fig. 1A), a naturally active flavonoid from the root of *Alpinia officinarum Hance*, has long been used as a folk remedy in Asian cultures for a variety of symptoms [Sohn et al., 1998]. It possesses various biological activities including antioxidant and radical scavenging activities [Russo et al., 2002], antimutagenic, anticlastogenic effect, and modulation of cytochrome P450 enzymes [Tiong et al., 2010]. Recent evidences indicated that galangin could inhibit several cancer cell lines proliferation and induce apoptosis. However, there is no insight into its influence on metastasis of melanoma.

In the present study, we investigated the effect of galangin on metastatic melanoma. For this purpose, highly metastatic B16F10 melanoma cell line was selected. Our results showed that galangin inhibited B16F10 cell proliferation, cell adhesion, cell spreading, and motility in vitro. FAK may be implicated in the antimetastatic effect of galangin, which has not been addressed before. In line with in vitro observations, evidence is also provided that galangin effectively inhibited formation and growth of metastatic loci in vivo.

MATERIALS AND METHODS

MATERIALS

Galangin was purchased from Guangzhou Institute for Drug Control (Guangzhou, China) and dissolved in dimethyl sulfoxide (DMSO) as a stock solution at 100 mM. The stock solution was kept at −20°C. High glucose Dulbecco's modified Dulbecco's medium (DMEM), fetal bovine serum (FBS), 0.05% trypsine–EDTA, a penicillin–streptomycin mixture (100×), lipofectamine 2000, the Prestoblue™ cell viability reagent, TRIzol reagent, fibronectin, and the superscript III first strand synthesis system were obtained from Invitrogen (Carlsbad, CA). An actin tracker green and a crystal violet staining solution were from Beyotime Institute of Biotechnology (Nanjing, China). The Millicell hanging cell culture insert with 8 μm pore PET membrane was purchased from Millipore (Billerica, MA). GelRed nucleic acid gel stain was from Biotium (Hayward, CA). The dual luciferase assay kit was from Promega (Madison, WI).

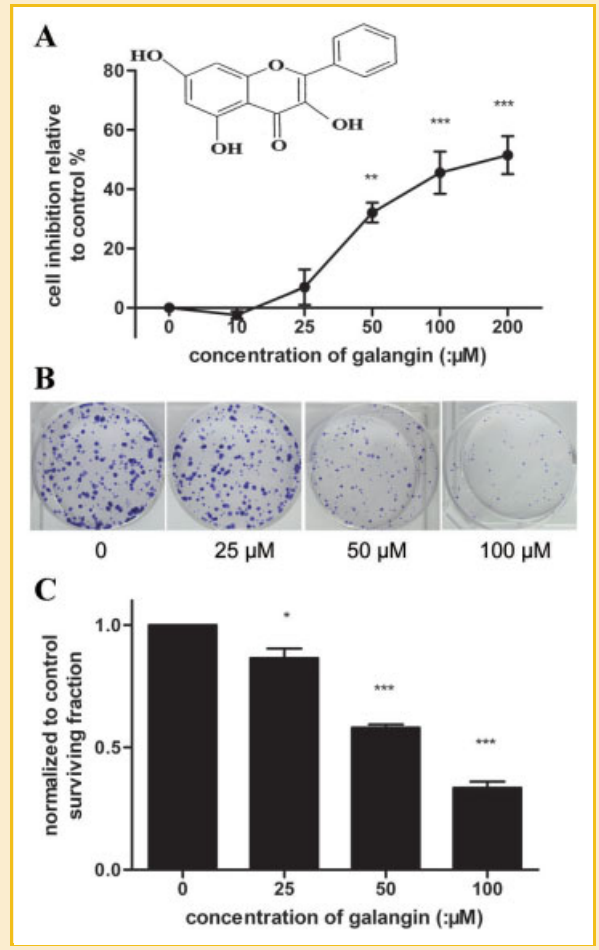

Fig. 1. Chemical structure and influence of galangin on B16F10 cell proliferation. A: Chemical structure of galangin and the Prestoblue™ assay showed suppression of cell growth in B16F10 cells. B16F10 melanoma cells were cultured in various concentrations of galangin for 24 h. Cell cytotoxicity was performed as described in Materials and Methods Section. Results are expressed as percentage of inhibition of growth compared with control, $^{**}P < 0.01$ and $^{***}P < 0.001$. B: Representative photographs of clonogenic formation. C: Quantitative analysis of the fraction of clonogenic survival assay. B16F10 cells were treated with galangin (0. 25, 50, and 100 μM) for 24 h and cultured for additional 7 days. At the end of incubation, cells were fixed with 3.7% paraformaldehyde and stained with the crystal violet solution. The surviving fraction was analyzed. The experiments were repeated three times with similar results. $^{*}P < 0.05$ and $^{***}P < 0.001$ versus control group.

CELL LINE AND MICE

The murine melanoma cell line B16F10 was obtained from the American Type Culture Collection (Manassas, VA) and cultured in high glucose DMEM containing 10% FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin. The cells were grown at 37°C with 5% CO_2. For animal experiments, female C57BL/6J mice (6–8 weeks old) obtained from Comparative Medical Center of Yangzhou University (Yangzhou, China) were housed in plastic cages and given food and water ad libitum. The room in which the mice was kept was environmentally controlled at a temperature of 25 ± 2°C where humidity and light were also carefully monitored (12 h light/12 h dark cycle). Animal welfare and experimental procedures were

performed strictly in accordance with high standard animal welfare and other related ethical regulations approved by Nanjing University Animal Care and Use Committee [Project Numbers SYXK (Su) 2009-0017]. All efforts were made to minimize the animals' suffering and to reduce the number of animals used.

CYTOTOXICITY ASSAY

The cytotoxic activity of galangin was measured using the Prestoblue™ cell viability reagent according to the manufacturer's protocol. Briefly, cultured B16F10 cells were seeded at a density of 5×10^4 cells/ml into 96-well plates. After an overnight incubation to allow cell attachment, the medium was replaced by different concentrations (10, 25, 50, 100, and 200 μM) of galangin for 24 h. Each concentration was repeated in five wells. And then the Prestoblue™ reagent was added to each well to incubate with the cells for 1 h at 37°C. Data values were collected by detecting the fluorescence intensity at $\lambda_{ex} = 560$ nm, $\lambda_{em} = 590$ nm at the Gemini EM Microplate Reader (Molecular Devices; Sunnyvale, CA). Cell inhibition rate was calculated using the formula: 100% × (1 − fluorescence intensity of treatment group/fluorescence intensity of control group).

CLONOGENIC SURVIVAL ASSAY

Exponentially growing cells were harvested, counted (~300 cells), and seeded into 6-well plates. Cells were allowed to grow at 37°C with 5% CO_2 overnight. Then, cells were treated with different concentrations of galangin for additional 24 h. After incubation, the cells were rinsed with PBS and replaced by fresh medium containing 10% FBS. Relative survival was calculated from the number of single cells that formed colonies of >50 cells at the 7th day. The survival fractions (SF) were corrected for the plating efficiency (PE) of the appropriate controls. PE = (no. of colonies formed/no. of cells seeded) × 100%; SF = no. of colonies formed after treatment/(no. of cells seeded × PE) [Franken et al., 2006].

CELL ADHESION ASSAY

The cell adhesion assay was done as previously with some modifications [Kucik and Wu, 2005]. The 96-well plates were precoated with 10 μg/ml fibronectin at 4°C overnight. The wells were blocked with 2% bovine serum albumin (BSA) for 1 h at 37°C. Cells were cultured in DMEM for 12 h and detached. One hundred microliters of cell suspension (5×10^5/ml) with and without galangin were added into each wells and incubated for 1 h at 37°C. Each concentration was repeated in five wells. At the end of incubation, non-adherent cells were washed away. The remaining cells were fixed with 3.7% paraformaldehyde and stained with the crystal violet solution for 15 min. Incorporated dye was solubilized using 1% sodium dodecyl sulfate (SDS). Relative cell adhesion was quantified by monitoring the absorbance of dye at 570 nm. As a negative control, cells were tested for adhesion to wells without fibronectin.

CELL SPREADING ASSAY

The cell spreading assay was carried out as cell adhesion assay. Briefly, the cells treated with different concentrations of galangin were seeded in 24-well plates. After 1 h incubation at 37°C, the cells were washed with PBS, fixed with 3.7% paraformaldehyde, and stained with crystal violet for 15 min. The stained cells were viewed under microscope and photographed (400×, Olympus ix 71, Japan). Spreading cells are defined as large cells with extensive membrane protrusions, medium type cells with moderate membrane protrusions, whereas non-spreading cells were represented as small, round cells with no membrane protrusions. Spreading, medium type and non-spreading cells were counted in five representative fields.

FLUORESCENCE ANALYSIS OF F-ACTIN

To visualize the actin cytoskeleton, cells were seeded in 24-well plates precoated with fibronectin. After 2 h incubation with serum free medium, in the presence of 10% FBS, 10% FBS medium and 50 μM galangin, the cells were fixed with 3.7% paraformaldehyde, and premeabilized in 0.1% Triton X-100 in PBS for 10 min. Phalloidin conjugated to FITC in a solution containing 0.1% Triton X-100 in PBS and 5% BSA was added to stain F-actin. After incubation for 30 min at room temperature, the cells were extensively washed to reduce nonspecific interactions. The cells were imaged by using the fluorescence microscope (400×, Olympus ix 71, Japan).

WOUND HEALING ASSAY

Wound healing assay was done as previously [Liang et al., 2007]. B16F10 cells were grown to confluent monolayers, which had been serum starved for 12 h. A 200 μl pipette tip was drawn across the center of the well to produce a clean wound area and the wounded cell layer was washed with fresh medium to remove loose cells. Immediately after wounding and at indicated incubation time (12 and 24 h) at 37°C in the presence or absence of 50 μM galangin, the images of the wound healing process were photographed digitally (100×). The gap distance normalized to control level was expressed at 0, 12, and 24 h considered.

MIGRATION ASSAY

The Millicell hanging cell culture insert with 8 μm pore PET membrane was used to conduct the migration assay. The lower chamber was filled with 900 μl of DMEM containing 10% FBS. Two hundred microliters cells suspension (5×10^4/ml) containing 50 μM galangin were added into the insert. The cells were allowed to migrate at 37°C with 5% CO_2 for 24 h. Cells that migrated to the lower surface of the inserts were washed, fixed, and stained with crystal violet. Quantitative OD 570 nm of crystal violet staining dissolved in 10% acetic acid represents the migrated cells.

REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION ASSAY (RT-PCR)

Total RNA was extracted from B16F10 cells after galangin treatment for 24 h using TRIzol reagent and the concentration of RNA was quantified by spectrophotometry. One microgram of total RNA was reverse transcribed into cDNA using the Superscript III first strand synthesis system according to the protocol of the manufacturer. The primers for murine FAK were 5′-TTATCCCAGCCCACAGCACAT-3′ (forward) and 5′-CCAGGGCGAGGAGGTTTCTT-3′ (reverse), primers for murine GAPDH were 5′-GGCAAATTCAACGGCACAGT-3′ (forward) and 5′-CGCTCCTGGAAGATGGTGAT-3′ (reverse). PCR was

performed in a Dyad® Peltier Thermal cycler PCR system. Aliquots (5 μl) of the amplification products were separated by 2% agarose gel electrophoresis and were visualized by GelRed nucleic acid gel stain under UV light using a molecular imager Chemidoc XRS+ system (Bio-Rad Laboratories, USA).

WESTERN BLOT ANALYSIS

Cells were treated with indicated concentrations of galangin for 24 h. Proteins were extracted with lysis buffer: 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton-X 100, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 10 mM NaF, 1 mM sodium vanadate (Na_3VO_4), 10% glycerol, and 1 mM EGTA. The lysates were cleared by centrifugation at 12,000 rcf for 30 min at 4°C. Protein concentrations were determined using the Bradford assay. For immunoblot, equal amounts of protein were electrophoresed on 10% SDS–PAGE gels and transferred to nitrocellulose. The proteins were probed with primary antibodies to phosphor-FAK (Tyr397; Cell Signaling Technology), FAK (BD Transduction Laboratories), and GAPDH (Santa Cruz Biotechnology). IRDye Rdye 800CW anti-mouse and anti-rabbit IgG were purchased from GE healthcare. Signal detection after secondary antibody incubation was done using odyssey infrared system (Li-Cor Biosciences).

TRANSIENT TRANSFECTION AND FAK LUCIFERASE REPORTER ASSAYS

Transfection of B16F10 cells was carried out using lipofectamine 2000 following the manufacture's instruction. Briefly, B16F10 cells were seeded into a 24-well plate at 1×10^5/well. Cells were cotransfected with 1 μg of pGL-FAK, a plasmid containing FAK promoter, and 1 μg of pRL-SV40 (Promega) as an internal control. Transfection was followed by rinsing and incubation in DMEM medium for 6 h. After the medium was changed, cells were allowed to recover for 18 h and then exposed to different concentrations of galangin for 24 h. Luciferase assays were performed using the Dual-luciferase Reporter Assay System according to the manufacturer's instructions. Briefly, 10 μl luciferase substrate was added to the 10 μl lysate, and luciferase activity was measured using a TD-20/20 Turner Designs Luminometer (Turner Designs, CA). Each luciferase assay was performed in triplicate.

TAIL VEIN METASTASIS AND IMMUNOHISTOCHEMISTRY

The cultured B16F10 cells were washed with and resuspended in serum free medium. Portions of cell suspension (5×10^5 cells/0.2 ml) were injected intravenously via the tail of C57BL/6J mice. A total of 16 mice were randomly divided into the control group (eight mice) and the treatment group (eight mice). Treatment was started 3 day after implantation. The mice of treatment group were administrated intraperitoneally with galangin at dose of 50 mg/kg/per day for consecutive 14 days. The control group received solvent at an equal volume. The mice were monitored by measuring body weight and by general clinical observation. Eighteen days later, the mice were euthanized. The lungs were resected and photographed. The numbers of metastatic nodules on the surface of lungs were counted macroscopically.

Tissues were stained for FAK expression, as described previously [Li et al., 2007]. Briefly, the tumors were fixed with 3.7% paraformaldehyde and embedded in paraffin. Five micrometers thick sections were prepared. Tumor sections were probed with FAK (Epitomics, diluted 1:50) primary antibodies, and then incubated with biotinylated secondary antibody followed by horseradish peroxidase-labeled streptavidin, and visualized with DAB chromogen (Sigma). Finally, the sections were counterstained with hemotoxylin. The sections were observed under a microscope (magnification, 100× and 400×). Five fields per slide and three slides per group were randomly selected.

STATISTICAL ANALYSIS

Results are expressed as mean ± SEM, each experiment was repeated three times. Data were statistically analyzed using one-way ANOVA followed by post hoc Dunnett's multiple comparison test. For in vivo experiments, the Mann–Whitney test was used for two group comparisons (Graphpad Prism Software, San Diego, CA). Statistical probability of $P < 0.05$ was considered statistically significant.

RESULTS

GALANGIN INHIBITS THE PROLIFERATION OF B16F10 CELLS

To examine the effect of galangin on the proliferation of B16F10 cells, we adopted two different techniques. Cells were incubated with different concentrations (10–200 μM) of galangin. The Prestoblue™ assay was used to detect cell viability. Galangin decreased the proliferation of B16F10 cells in a dose-dependent manner. The cell viabilities were 67.9% at 50 μM group, 54.5% at 100 μM group, and 48.7% at 200 μM group, respectively (as shown in Fig. 1A).

The ability of cells to form colonies is positively correlated with proliferation and metastatic potential. After exposure to galangin for 24 h, as shown in (Fig. 1B,C), untreated B16F10 cells formed sizeable colonies and showed rapid proliferation. However, galangin-treated B16F10 cells showed reduced efficiency to form colonies. For example, the clonogenic survival fraction was reduced from 85.1% to 33.4% of the control values for 25–100 μM galangin treated groups.

GALANGIN NEGATIVELY REGULATES FIBRONECTIN MEDIATED CELL ADHESION AND SPREADING WITH F-ACTIN CYTOSKELETON ARRANGEMENT

As cell adhesion is one of the essential steps involved in cancer metastasis, we investigated the effect of galangin on cancer cell adhesion. B16F10 cells were allowed to adhere to fibronectin for 1 h in the presence of 25–100 μM galangin. As shown in Figure 2A, galangin negatively regulated cell adhesion to fibronectin dose-dependently. Cell spreading is another common approach to assess cell adhesion. Relative quantification as present in Figure 2B indicated that the spreading cells in the 25, 50, and 100 μM groups were 54.2%, 4.77%, and 0.94% of the control group ($P < 0.05$). Galangin significantly suppressed cell spreading on fibronectin. The presence of fibronectin significantly enhanced cell adhesion compared with BSA. Most cells exhibited spreading morphology after 1 h of plating in control group, whereas the cells became defects in polarized extension and remained rounded after galangin treatment (Fig. 2C). Reorganization of F-actin filaments is essential in adhesion and migration to adapt the cell shape to the surrounding

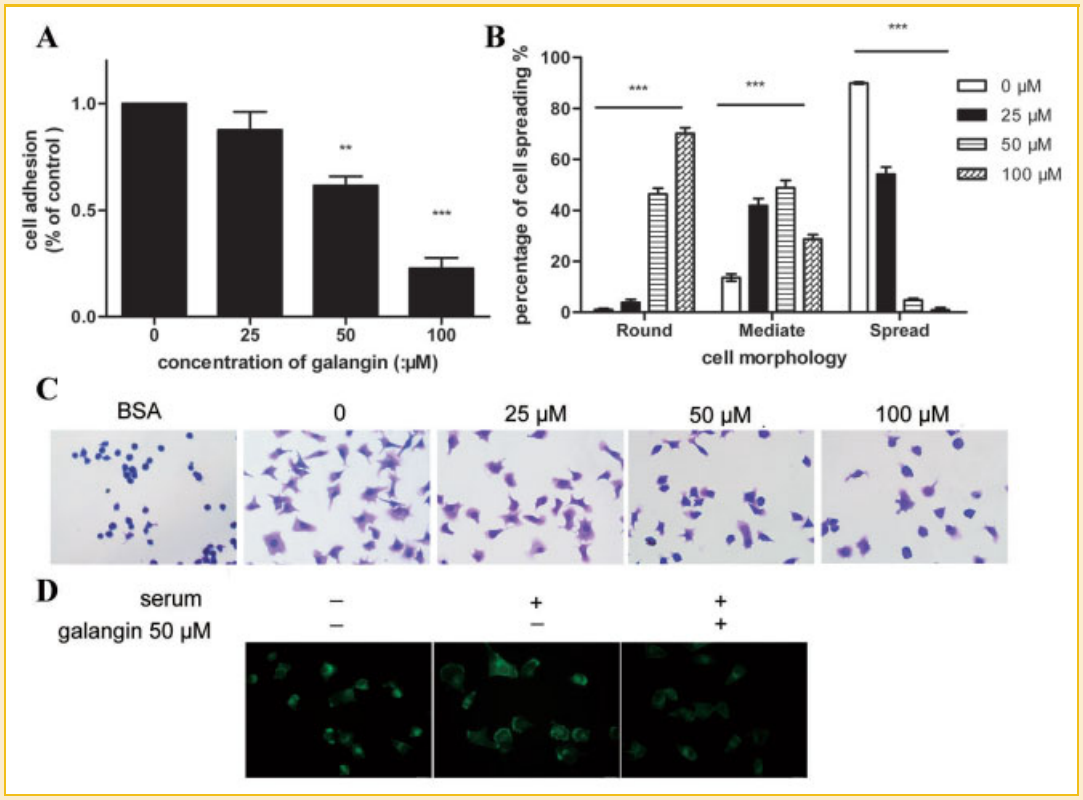

Fig. 2. Effects of galangin on B16F10 cell-substratum interactions on fibronectin. A: Inhibitory effect of galangin on B16F10 cell adhesion on fibronectin. Cells were serum-starved for 12 h and then plated onto fibronectin-coated 96-well plates with 25, 50, and 100 μM galangin for 1 h. Adhesive cells were stained with crystal violet and dissolved in 1% SDS. The numbers of adhesive cells were evaluated by the absorbance values at 570 nm wavelength. The rate of inhibition of cell adhesion was normalized to control level. $^{**}P < 0.01$ and $^{***}P < 0.001$. B: Suppressing effect of galangin on B16F10 cell spreading on fibronectin. Cells were serum-starved for 12 h and then plated onto fibronectin-coated 24-well plates with indicated galangin for 1 h. Cells were stained with crystal violet and at least 200 cells per group were counted. The percentage of round, medium and spread cells were shown. Quantitative data based on three independent experiments, $^{***}P < 0.001$ versus individual control. C: Images of inhibitory effect of galangin on spreading of B16F10 cells to fibronectin (magnification, 400×), the cells in the wells without fibronectin precoated were used as negative control (BSA group). D: Fluorescence staining of F-actin in B16F10 cells (magnification, 400×). Cells were serum-starved for 12 h and then plated onto fibronectin coated 24-well plates for 2 h in starvation medium, in the presence of 10% FBS, in the presence of 10% FBS and 50 μM galangin. F-actin was stained with FITC-conjugated phalloidin. Data are representative of three independent experiments.

microenvironment. We proceeded to investigate the cytoskeleton change by staining F-actin. Our results showed that F-actin in serum starved B16F10 cells was mainly surrounded the nuclear, whereas the presence of serum induced the formation of lamellipodia at the leading edge, addition of 50 μM galangin disrupted the formation of lamellipodia (Fig. 2D).

GALANGIN RETARDS B16F10 CELLS MOTILITY AND MIGRATION

Since cell adhesion is an important component of cell migration, the observed effect of galangin on the B16F10 cell adhesion indicated that it might also affect cell migration. Increased cell motility is another characteristic associated with malignancy. As shown in Figure 3A,B, using wound healing assay, we found that galangin significantly slowed the rate of cells into the wounded area compared to the control group at 12 and 24 h (control group: 73.3% and 39.3%; 50 μM group: 82.3% and 79.3%, respectively) Furthermore, we utilized Millicell hanging cell culture insert to check the migration ability of B16F10 cells with 50 μM galangin in a three dimensional mode. A significant reduced number of migrating cells was observed when the cells were treated with galangin for 24 h (35.4% in 50 μM galangin vs. control, $P < 0.001$; Fig. 3 C).

GALANGIN REDUCES FAK EXPRESSION IN B16F10 CELLS

The overexpression of FAK correlates with motility of human melanoma cells. Constitutive activation of FAK is important for aggressive melanoma growth. To determine whether galangin reduced cell adhesion and migration through regulation of FAK, semi-quantitative RT-PCR was employed to examine the transcripts of FAK. Cells were treated with galangin (0, 25, 50, and 100 μM) for 24 h. Major inhibition of FAK mRNA occurred dose-dependently, confirming that down modulation involved transcriptional regulation of FAK gene (Fig. 4A,B). To illustrate whether the transcriptional inhibition of FAK lead to reduction of FAK protein by galangin, the expression of FAK was examined using Western blot analysis. Galangin suppressed FAK total protein level from 25 to 100 μM (Fig. 4C). Additionally, the phosphorylation status of FAK Tyr397 was also analyzed. Constitutive activation of phosphor-FAK was detected in the B16F10 cells without galangin. Galangin markedly reduced the activation of FAK. To further verify the

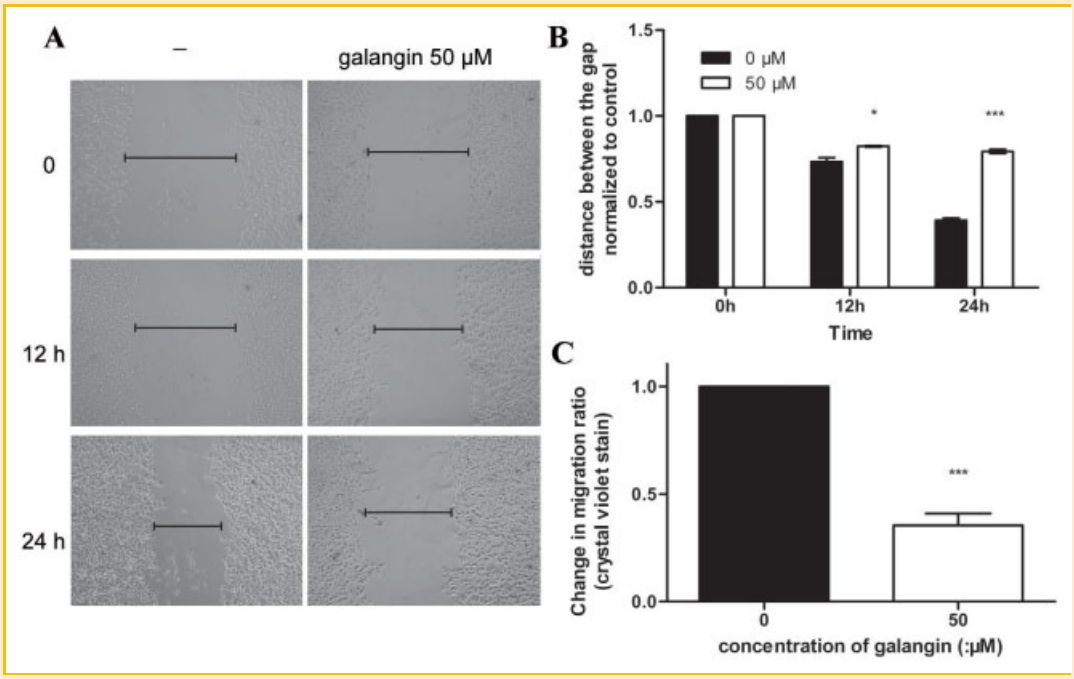

Fig. 3. Galangin suppresses motility and migration in B16F10 melanoma cells. A: Microscopic appearance of wound healing processes of control and B16F10 cells treated with 50 μM galangin at 0, 12, and 24 h. B16F10 cells were plated in 24-well plates and grown to 80% confluence. After generating a scratch in the monolayer, cells were incubated with and without 50 μM galangin. Photographs were taken immediately, 12 and 24 h after wounding (magnification, 100×). B: The area of gap region was quantified by measuring four selected fields under the microscope, $^{*}P < 0.05$ versus untreated group at 12 h, $^{***}P < 0.001$ versus untreated group at 24 h. C: Galangin decreased B16F10 cell migration in the Millicell hanging cell culture insert. B16F10 cells treated with and without 50 μM galangin were seeded in the upper chamber of millicell hanging insert. After 24 h of incubation, cells that migrate to the lower surface of the filter were fixed, stained, and rinsed in 10% acetic acid. The quantitative absorbance values at 570 nm wavelength represented migrated cells. The results shown are representative of three independent experiments, $^{***}P < 0.001$ versus untreated group.

modulation of galangin on FAK gene transcription, we construct FAK promoter luciferase reporter plasmid and performed dual luciferase assays. The firefly luciferase expression is driven by a FAK response element located upstream of a basal SV40 promoter, and the control reporter pRL-SV40-renilla, in which renilla luciferase expression was driven by the SV40 promoter alone. The B16F10 cells transiently transfected with the constructed plasmid were treated with 25–100 μM galangin for 24 h. Cell lysates were harvested for luciferase assays. The results shown in (Fig. 4D) indicated that the ratio of pGL-FAK/pRL-SV40-renilla activity, which is a measure of FAK transcriptional activity, was 7.88 ± 1.10 in control group down to 1.11 ± 0.07 in 100 μM galangin group.

GALANGIN INHIBITS LUNG METASTASIS OF B16F10 CELLS IN VIVO

To determine whether the anti-metastasis effect of galangin against B16F10 melanoma cells can be reproduced in vivo, we performed the tail vein metastasis model. The injection of B16F10 cells into the vein of mice induced lung metastasis. As shown in Figure 5A, treatment of the mice with galangin (50 mg/kg) reduced the number of nodules per mouse ($P = 0.007$, $P < 0.01$). The mean number of metastatic lung nodules per lung was 7.6 ± 1.5 for control group and 3.4 ± 2.4 for the treatment group. Both groups were gaining weight at the 18th day, and this trend was more evident in the control group (Table I). Moreover, there are four out of eight mice were found abdominal metastasis in control group and none in galangin group. Meanwhile, tumor tissues were taken and paraffin-embedded sections were prepared for immunochemical analysis. The in vivo sections clearly showed that the tissues in the PBS-treated mice expressed high level of FAK in cytoplasm, whereas FAK expression was reduced in galangin-treated mice (Fig. 5B).

DISCUSSION

Metastasis represents the final, most devastating stage of malignant melanoma. Current median survival for metastatic melanoma patients is about 6–9 months. Chemotherapy, immunotherapy and surgical therapy were achieved little progresses [Gogas et al., 2007]. In 2011, the US Food and Drug Administration (FDA) approved two novel therapies for advanced melanoma: vemurafenib and ipilimumab. Although they have their limitations, there is no doubt that they bring new hope to physicians and patients. It is expected that rational combination with other agents targeting therapeutic biomarkers will hopefully lead to further treatment advances [Amaria et al., 2012; Finn et al., 2012]. The research and development of novel anti-melanoma agents from phytochemicals is one of the most attractive fields [Heng, 2010; Lee et al., 2012]. However, rigorous and systematic in vitro and in vivo pre-clinical evaluations are urgently needed for phytochemicals [Ulbricht and Chao, 2010].

Previously reports have demonstrated that galangin inhibited proliferation as well as induction of apoptosis in several cancer cell

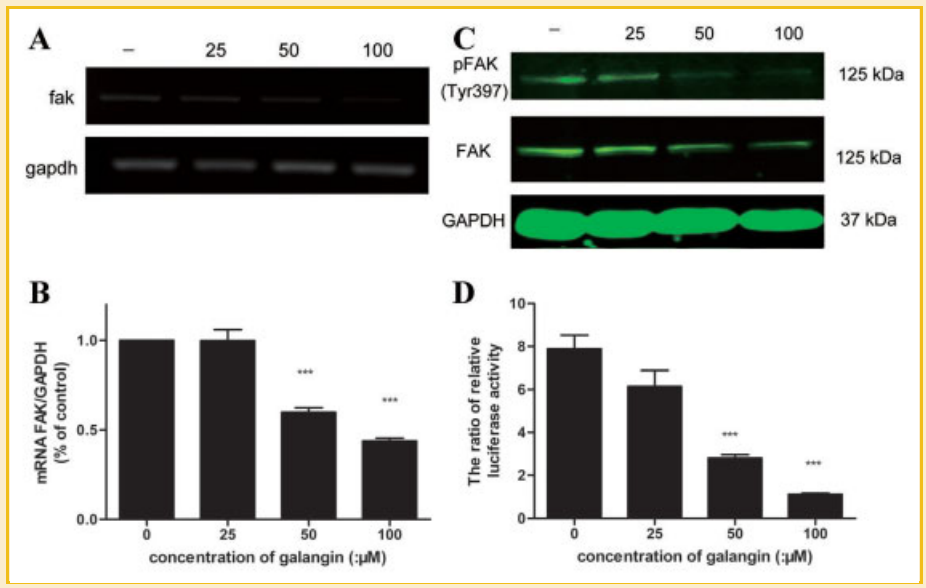

Fig. 4. The galangin-induced anti-metastatic effect is dependent on FAK expression. A: FAK transcripts of galangin-treated B16F10 cells were validated using reverse transcription PCR. Cells were treated with indicated galangin for 24 h. Total RNA was isolated and analyzed by RT-PCR, GAPDH was used to show equal loading of total RNA. Representative agarose of FAK was shown. B: Quantification of FAK mRNA expression. The experiments were performed at least three times, $^{***}P < 0.001$ versus control. C: FAK and its phosphorylation form (Tyr397) in galangin-treated B16F10 cells. After cells were treated with 0, 25, 50, and 100 μM galangin, the cell lysates were subjected to Western blotting analysis with antibody against phosphor-FAK, the same blot was stripped and reprobed with FAK antibody. GAPDH was probed to show equal protein loading. Immunoblot images are from a representative experiment. The experiments were performed in triplicate. D: Galangin modulated FAK promoter activity. B16F10 cells co-transfected with pGL-FAK promoter luciferase reporter construct and pRL-SV40 were treated with galangin as descried in Materials and Methods Section. FAK promoter activity was evaluated by the ratio of relative luciferase activity, $^{***}P < 0.001$ versus the untreated group.

lines. It is recognized as a promising cancer chemopreventive agent [Heo et al., 2001]. Galangin effectively inhibited the proliferation of an ER^- cell line accompanied by down regulation of cyclin D3, E, and A [Murray et al., 2006]. It is reported that galangin promoted hepatocellular carcinoma cells apoptosis via the mitochondrial pathway, such as collapse of the mitochondrial membrane potential, bax translocation, release of cytochrome C, and activation of caspase cascades [Zhang et al., 2010]. Gwak et al. [2011] reported

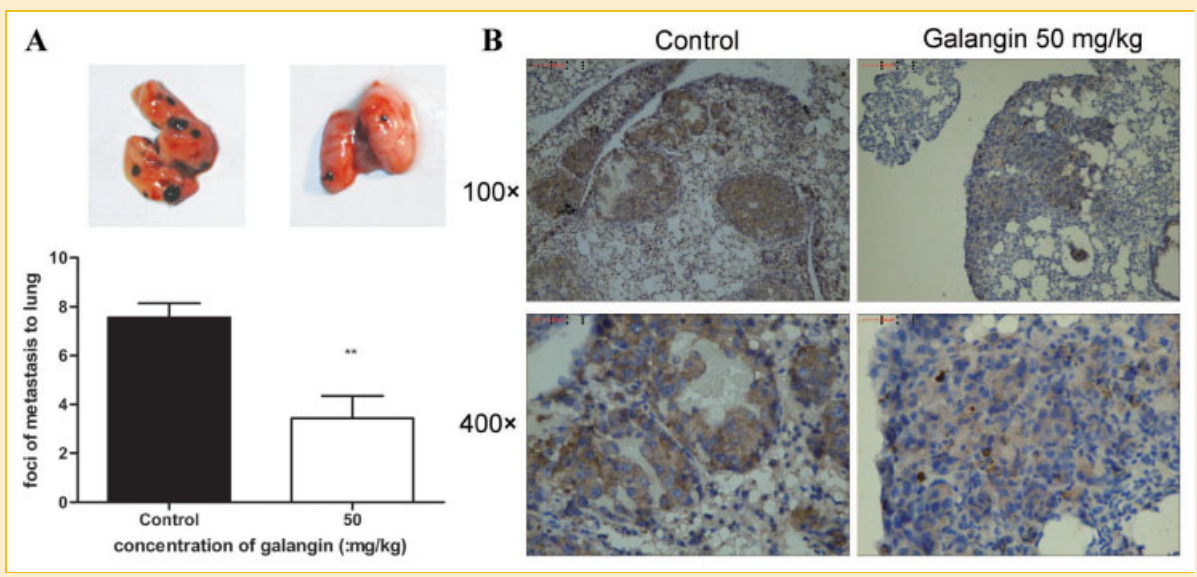

Fig. 5. Galangin suppresses B16F10 cell lung metastasis and FAK expression in vivo. Cell suspension (5×10^5 cells/0.2 ml) was injected intravenously via the tail of C57BL/6J mice. Mice were treated with a solvent and 50 mg/kg galangin as described in Materials and Methods Section. A: Gross appearance of typical lungs dissected from control and galangin 50 mg/kg mice was shown in upper panel. The number of experimental lung metastases was quantified as in the lower panel. Each group contained eight mice, $^{**}P < 0.01$ versus control group. B: Immunohistochemical analyses were performed using antibody against FAK. Original magnification: 100× and 400×, respectively.

TABLE I. Experimental Cure of Galangin to the Lung Metastasis Models of Mice B16F10 Cells

Treatment	Dose	Animals		Foci of metastasis	*P*-value for incidence of metastasis
		Number	Weight		
PBS	–	8/8	18.2[a]/19.1[b]	7.6 ± 1.5	$^{**}P < 0.01$
Galangin	50 mg/kg	8/8	17.8[a]/18.4[b]	$3.4 \pm 2.4^{**}$	

[a]Before the experiment.
[b]After the experiment.

that galangin mediated β-catenin degradation and suppressed the proliferation of CRT-positive cancer cell proliferation. However, no information is available on the influence of galangin on metastasis. The purpose of this study was to investigate the effect of galangin on the metastatic potential of B16F10 cells in vitro and in vivo, and to gain primary insight into the underlying mechanism.

The formation of distant metastasis involves several sequential steps, including tumor growth in the primary site, invasion into blood vessels, arrest in the capillaries, extravasation, invasion and growth in target organs [Bacac and Stamenkovic, 2008]. Failure at any of processes could block the metastasis. Given that growth of metastases is a rate limiting step during metastasis [Chambers et al., 2002; Gupta and Massague, 2006], we first demonstrated the cytostatic effect of galangin in B16F10 cells by cell viability and clonogenic survival assays. Cancer cell attachment via cell surface receptors that specifically bind to the matrix components such fibronectin or lamin is necessary for circulating cancer cells adhesion to the vasculature. Our finding indicated galangin impaired fibronectin-induced B16F10 melanoma cell adhesion and spreading. Cell matrix adhesion could regulate actin cytoskeleton organization, such as formation of filopodia or lamellipodia [Defilippi et al., 1999]. Indeed, the formation of lamellipodia at the leading edge was inhibited in galangin treated B16F10 cells. Fundamentally, metastasis involves the movement of cancer cells from one site to another. Both of wound healing and migration assay showed that 50 μM galangin impaired B16F10 cell motility. The in vivo experiments further confirmed that galangin (50 mg/kg) resulted in a dramatic reduction of metastasis of melanoma cells to the lung without adversely affecting body weight.

FAK, an intracellular tyrosine kinase, coordinates all processes that involved in the development of cancer [Luo and Guan, 2010]. The link between FAK and cell survival was first revealed by Frisch et al. [1996] showing that constitutively activated forms of FAK rescued two established epithelial cell lines from anoikis. Treatment with antisense FAK oligonucleotides reduced FAK expression and increased cell apoptosis and sensitivity to the chemotherapy drug 5-fluorouracil in human melanoma cells [Smith et al., 2005]. Hess and Hendrix [2006] have shown that the tyrosine residues (397 and 576) were important in aggressive melanoma cells on a three dimensional type I collagen matrix in vitro as well as in radial and vertical growth phase melanomas in situ. Expression of FRNK in melanoma resulted in the inhibition of the aggressive phenotype. In our molecular data, we showed that reduced FAK at the mRNA and protein level in B16F10 melanoma cells derived from galangin treatment, as well as the activity of FAK phosphorylation (Tyr397). Reduced FAK expression and activity contribute to decrease in B16F10 cell proliferation, adhesion, and motility implicated in galangin effect. Previously, Huang et al. [2005] reported that flavonoids (luteolin and quercetin) effectively lead to reduced motility and invasion of MiaPaCa-2 cells through inhibition of FAK phsophorylation and expression by EGFR. The reduced phosphorylation of FAK observed in our study may accord with disrupting EGFR-mediated tyrosine kinase activity by galangin. Transcription regulation of FAK, especially in metastatic melanoma seems promising for high copies of FAK gene expression in malignant melanoma [Li and Hua, 2008]. Agents suppressing FAK gene would have little or no effect on normal cells. A recent study demonstrated that a correlation between FAK mRNA level and FAK promoter luciferase activity [Golubovskaya et al., 2004]. We indeed found that galangin could suppress FAK transcription in our constructed FAK promoter luciferase reporter, consistent with decrease level of fak mRNA. Collectively, FAK is a potential drug target of galangin, regulating at both gene transcription and protein phosphorylation levels. In addition, the immunostaining of FAK expression was decreased significantly in the galangin treated group in vivo. However, the exact mechanism of galangin in this regulation of fak promoter remains to be determined in our further study.

Recent evidence suggesting flavonoids inhibit cancer invasion and metastasis through interaction with NF-κB is increasingly in the scientific literature. These include curcumin [Gupta et al., 2010], resveratrol [Liu et al., 2010], genistein, apigenin, luteolin [Lin et al., 2008], and kaempferol [Lin et al., 2010]. These agents suppress NF-κB activation through various levels. For example, curcumin blocks IKK activation [Aggarwal et al., 2005], resveratrol suppresses p65 phosphorylation [Manna et al., 2000], genistein mediates downregulation of NF-κB DNA-binding activity [Li and Sarkar, 2002], and apigenin down regulates the nuclear factor (NF)-κB subunit p65 [Liao et al., 2012]. On the other hand, NF-κBs play important roles in the tumor progression as well as tumor metastasis. Activation of NF-κB has been observed in many cancers, including but not limited to melanoma [Chaturvedi et al., 2011]. NF-κB transcription factors contains: RelA/p65, c-Rel, RelB, p50/NF-κB1, and p52/NF-κB2, which dimerize within the cytoplasm by the inhibitors of NF-κBs (IκBs). The IκB kinase (IKK) complex, comprised of two catalytic (IKKα and IKKβ) and one regulatory (IKKγ/NEMO) subunits, is the immediate upstream of the IκBs. Several pathways of cell stimulation converge to activate the IKK complex, which then phosphorylates the IκBs, targeting them for ubiquitination and degradation [Naugler and Karin, 2008]. The released NF-κBs translocate to the nucleus and enhance transcriptional activation of NF-κBs dependent genes. FAK promoter is GC rich and has multiple potential transcriptional binding sites.

Importantly, FAK promoter region has NF-κB and p53 binding sites. Only NF-κB positively regulated FAK expression [Golubovskaya et al., 2004]. It appears reasonable to suggest that NF-κB might be the key link between galangin and the promoter of FAK gene.

In summary, our studies in this report demonstrate for the first time that galangin has a novel pharmacological function of anti-metastasis, and FAK is probably its new drug target for this anti-metastatic function. Galangin effectively inhibited cell proliferation, adhesion, spreading, motility, and lamellipodia formation in vitro, which are required for tumor metastasis. Treatment with galangin resulted in the suppression of B16F10 melanoma experimental metastasis in vivo. The cellular mechanism of galangin rests on a previously unidentified role in negatively regulating transcription of FAK gene. Further in depth research for the key transcriptional factor regulated by galangin is needed. It is expected that galangin may serve as an efficient anti-metastasis herbal medicine in melanoma treatment.

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