

Ginsenoside Rg5 inhibits cancer cell migration by inhibiting the nuclear factor- κ B and erythropoietin-producing hepatocellular receptor A2 signaling pathways

LEIXIN SONG¹, FAN YANG¹, ZHENGTAO WANG^{1,2}, LI YANG^{1,2} and YUE ZHOU^{1,3}

¹The MOE Key Laboratory for Standardization of Chinese Medicines and The SATCM Key Laboratory for New Resources and Quality Evaluation of Chinese Medicines, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine; ²Shanghai R&D Centre for Standardization of Chinese Medicines, Shanghai 201203, P.R. China; ³Department of Cancer Cell Biology, Faculty of Pharmaceutical Sciences, University of Toyama, Toyama 9300194, Japan

Received May 7, 2020; Accepted December 17, 2020

DOI: 10.3892/ol.2021.12713

Abstract. The majority of cancer-associated deaths are caused by cancer metastasis, the first step of which is the acquisition of migratory ability by cancer cells. Therefore, the suppression of cancer cell migration represents a potential efficient strategy to inhibit cancer metastasis. Inflammation induces cancer cell migration through the activation of nuclear factor- κ B (NF- κ B), which is a transcription factor that serves a central role in inflammatory signaling. Recent studies have demonstrated that the phosphorylation of the receptor tyrosine kinase erythropoietin-producing hepatocellular receptor A2 (EphA2) at S897 promotes cancer cell migration. Therefore, a compound with the ability to abolish these two factors may suppress cancer metastasis. In the present study, ginseng saponin ginsenoside Rg5 was found to inhibit the phosphorylation of NF- κ B and EphA2. Therefore, this study aimed to elucidate the molecular mechanisms of ginsenoside Rg5 and determine whether it inhibited cancer cell migration. The results demonstrated that ginsenoside Rg5 inhibited the activation of NF- κ B by suppressing its upstream kinase transforming growth factor β -activated kinase 1 in TNF- α treated HeLa or A549 cells compared with that in the untreated control group. Furthermore, ginsenoside Rg5 attenuated the expression of EphA2 by lysosomal degradation, which

inhibited its phosphorylation. In addition, ginsenoside Rg5 suppressed inflammatory cytokine-induced cancer cell migration. In conclusion, the results of the present study provided a scientific basis for the development of ginsenoside Rg5 as a potential antimetastatic drug.

Introduction

Metastasis is responsible for ~90% of cancer-related deaths (1). Metastasis is a multi-step process that begins with the acquisition of migratory ability by tumor cells (1). Therefore, the suppression of tumor cell migration may represent an efficient strategy for the inhibition of cancer metastasis. Inflammation is a critical factor for tumor malignancy, including cancer cell migration and tumor metastasis (2-5). The transcription factor nuclear factor- κ B (NF- κ B) serves an essential role in inflammatory signaling (3). Upon a stimulation by inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β), transforming growth factor β -activated kinase 1 (TAK1) is phosphorylated and induces the activation of I κ B kinases (IKKs) (4). IKKs subsequently phosphorylate p65, the major component of NF- κ B, and I κ B α , the inhibitor of p65. At the same time, phosphorylated I κ B α is ubiquitinated and transferred to the ubiquitin-proteasome system for degradation (4). Phosphorylated p65 then translocates from the cytosol into the nucleus to activate the transcription of genes that promote cell migration, such as matrix metalloproteinases and epithelial-to-mesenchymal transition-inducible genes (3-5). Therefore, the suppression of p65 activation may be an effective strategy for inhibiting cancer metastasis.

Erythropoietin-producing hepatocellular receptor A2 (EphA2) belongs to the receptor tyrosine kinase family and maintains intercellular adhesion in normal cells (6,7). However, it is upregulated in various types of malignant tumors, such as lung and colorectal cancer, glioblastoma and melanoma, particularly in metastatic tumors (6,7). Previous studies have demonstrated that ligand- and tyrosine kinase-independent EphA2 phosphorylation at S897, which is induced by the activation of p90 ribosomal S6 kinase (RSK), controls cancer

Correspondence to: Dr Yue Zhou or Dr Li Yang, The MOE Key Laboratory for Standardization of Chinese Medicines and The SATCM Key Laboratory for New Resources and Quality Evaluation of Chinese Medicines, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, 1200 Cai Lun Road, Zhangjiang Hi-TechPark, Pudong New Area, Shanghai 201203, P.R. China
E-mail: yue_zhou_etsu_shu@126.com
E-mail: cpuyil@126.com

Key words: ginsenoside Rg5, nuclear factor- κ B, transforming growth factor β -activated kinase 1, erythropoietin-producing hepatocellular receptor A2, cancer migration

cell migration (6,8). In addition, the RSK-EphA2 axis has been implicated in the poor survival of patients with lung cancer, suggesting its potential as a novel molecular target for pharmacological interventions (8).

Ginseng is the rhizome of plants in the genus *Panax*, such as South China ginseng (*P. notoginseng*), Korean ginseng (*P. ginseng*) and American ginseng (*P. quinquefolius*), and is traditionally used to treat various diseases, such as inflammation, lung and colon cancer (9-11). Steaming and heating processes have often been adopted to alter or enhance the pharmacological activities of various natural medicines; steamed or heat-processed ginseng has frequently been used in the treatment of tumors (12), inflammation (13) and stress-related conditions (14,15). Certain compounds in processed ginseng, including ginsenoside Rh2 and Rg5, appear to exert inhibitory effects against cancer cell migration (12,16). Ginsenosides, also termed ginseng saponins, are the main bioactive ingredients of ginseng (15). Various ginsenosides have been reported to exert antimetastatic (17) and anti-inflammatory (16,18) effects. However, sufficiently detailed research on ginsenosides, particularly processed ginsenosides, at the molecular level has not been performed to date. The present study screened ginsenosides that exerted inhibitory effects on NF- κ B activity and identified ginsenoside Rg5. The aim of the present study was to elucidate the molecular mechanisms of ginsenoside Rg5 and determine whether it could block the migration of A549 cells.

Materials and methods

Antibodies and reagents. Antibodies against phospho-p65 (S536; cat. no. 3033), phospho-ERK (T202/Y204; cat. no. 4370), phospho-RSK (S380; cat. no. 11989), phospho-TAK1 (T187; cat. no. 4536), phospho-IKK α / β (S176/177; cat. no. 2697), phospho-EphA2 (S897; cat. no. 6347), phospho-EphA2 (Y577; cat. no. 12677), TAK1 (D94D7; cat. no. 5206), TAK1 binding protein 1 (TAB1) (C25E9; cat. no. 3226), TAB2 (C88H10; cat. no. 3745), IKK β (D30C6; cat. no. 8943), ERK1/2 (137F5; cat. no. 4695), EphA2 (D4A2; cat. no. 6997) and β -actin (D6A8; cat. no. 8457) were purchased from Cell Signaling Technology, Inc. Antibodies against p65 (C-20; cat. no. sc-372), IKK α (H-744; cat. no. sc-7218), I κ B α (C-21; cat. no. sc-371), RSK1 (C-21; cat. no. sc-231), RSK2 (C-19; cat. no. sc-1430) and Lamin B1 (8D1; cat. no. sc-56144) were obtained from Santa Cruz Biotechnology, Inc. Recombinant human TNF- α was obtained from R&D Systems, Inc., and recombinant human IL-1 β from PeproTech, Inc. Ginsenoside Rg5 was purchased from Biopurify Phytochemicals Ltd. and dissolved in dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA). Cycloheximide, MG-132 and bafilomycin A1 were purchased from Sigma-Aldrich; Merck KGaA, and the cells were treated for 2-6 h at 37°C.

Cell culture and treatment. HeLa, A549 and 293T cells were purchased from Shanghai Fan Shuo Biological Technology, and 293 cells were obtained from ATCC. These cells were maintained in high-glucose Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in 5% CO₂. A549 cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific,

Inc.) supplemented with 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in 5% CO₂.

Cells were stimulated with recombinant human TNF- α (10 ng/ml) or IL-1 β (10 ng/ml) for 24 h at 37°C with 5% CO₂. Ginsenoside Rg5 or an equal volume of DMSO was added for 30 min before the TNF- α or IL-1 β stimulation.

Luciferase assay. HeLa cells were transferred with a luciferase reporter plasmid provided by Professor Hiroaki Sakurai (University of Toyama, Toyama, Japan) under the control of four sites containing a neomycin resistance gene (19). A stable clone was isolated in DMEM containing 500 μ g/ml G418 (Thermo Fisher Scientific, Inc.). The transfected cells (5×10^4) were seeded in a 96-well plate and stimulated with TNF- α for 6 h at 37°C with 5% CO₂. Luciferase activity was measured using a Dual-Luciferase Reporter Assay system (Promega Corporation). The experiments were conducted in triplicate.

Immunoblotting. Whole-cell lysates of HeLa, A549, 293 and 293T cells were prepared using a whole-cell lysate buffer [1.0 M HEPES-NaOH, pH 7.7; 0.3 M NaCl; 0.1 M MgCl₂; 0.5 M EDTA, pH 8.0; 10% Triton X-100; and protease/phosphatase inhibitor cocktails (Roche Diagnostics)]. The concentration of lysates was measured by BCA assay (Thermo Fisher Scientific, Inc.), and each sample was adjusted to the same concentration. Each sample was mixed with an equal volume of SDS-PAGE sample buffer (195 mM Tris-HCl, pH 6.8; 3% SDS; 15% DTT; 30% glycerol; and 0.10% bromophenol blue) and heated at 95°C for 5 min. The samples (5-20 ng) were separated by 7.5 or 10% SDS-PAGE and transferred to Immobilon-P nylon membranes (EMD Millipore). The membranes were treated with SuperBlock (Thermo Fisher Scientific, Inc.) or BlockAce (KAC Co., Ltd.) overnight at 4°C and probed with primary antibodies (dilution, 1:1,000-2,000) for 2 h at room temperature. The antibodies were detected using a horseradish peroxidase-conjugated anti-rabbit, anti-mouse or anti-goat secondary antibody (dilution, 1:5,000; cat. nos. 7074 and 7076; Cell Signaling Technology, Inc.; and cat. no. PA1-28664; Thermo Fisher Scientific, Inc.) for 1 h at room temperature and visualized by Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore) on Amersham Imager 600 (Cytiva) or X-ray film. Analyses were performed using Adobe Photoshop CC (Ver. 14.2.1, Adobe, Inc.) or ImageJ (Ver. 1.52; National Institutes of Health) and GraphPad Prism 7 (Ver. 7.0.0.159; GraphPad Software, Inc.) at least three times, and representative images are presented.

Thermal shift assay. Samples of HeLa, A549 or 293T cells were prepared as described previously (20) and analyzed by immunoblotting as aforementioned.

Transfection of plasmid DNA. 293T and 293 cells at 50% confluency were transfected using Effectene Transfection Reagent (Qiagen, Inc.) and Lipofectamine[®] 2000 reagent (Thermo Fisher Scientific, Inc.), respectively, according to the manufacturer's instructions. Expression vectors for human TAK1, TAB1, TAB2, EphA2, RSK1 and p65 were provided by Professor Hiroaki Sakurai (University of Toyama, Toyama, Japan). The amount of plasmid DNA was 0.5 ng. After 24-h incubation at 37°C with 5% CO₂, the transfected cells were

used in subsequent experiments. Successful transfection was determined by immunoblotting.

Reverse transcription-quantitative (RT-q)PCR analysis. Total RNAs were extracted from HeLa and A549 cells using RNA FASTER200 reagent (Shanghai Fastagen Biotechnology Co., Ltd.) according to the manufacturer's instructions. RNA was reverse-transcribed to generate first-strand cDNAs using the PrimeScript™ RT Master Mix Kit (Takara Bio, Inc.); the reaction conditions were 37°C for 15 min and 85°C for 5 sec. The qPCR analysis was performed using the SYBR® Premix Ex Taq™ (Tli RNaseH Plus) Kit (Takara Bio, Inc.) according to the manufacturer's instructions on a 6000 Real-Time PCR System (Thermo Fisher Scientific, Inc.), and the relative expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (21). The cycle threshold values of the target genes were normalized to those of *GAPDH* from the same sample. The primer sequences were as follows: *EPHA2* forward, 5'-CCATCCATCCTGTGTCA-3' and reverse, 5'-TCGCTGCTTCTCTGTGT-3'; and *GAPDH* forward, 5'-GGGAAGGTGAAGGTCGGAGT-3' and reverse, 5'-GGGGTCATTGATGGCAACA-3'.

Wound healing assay. A549 or 293 cells were cultured in a 3.5 cm dish until they formed a monolayer, which was scratched using a 200- μ l pipette tip. Cells were washed to remove cellular debris, fresh medium with 10% FBS was added, and the cells were allowed to migrate for 24 h at 37°C with 5% CO₂. Due to the high toxicity of ginsenoside Rg5 and the low cell viability in presence of lower amounts of FBS, the assays were performed using complete medium. Images were captured by an Olympus CKX53 (magnification, x10; Olympus Corporation) or a Nikon Diaphot (magnification, x4; Nikon Instruments, Inc.) phase contrast microscope at the same position within the wound region at 0 and 24 h, and the distance of cell migration was measured using ImageJ. Analyses were performed at least three times and representative results are shown.

Migration assay. A migration assay was performed using Transwell chambers (Costar; Corning, Inc.), and the lower surface was pre-coated with 1.25 μ g fibronectin for 2 at room temperature (Sigma-Aldrich; Merck KGaA). A549 cells (2×10^5 cells/well) pre-treated with ginsenoside Rg5 and TNF- α or IL-1 β were added to the upper compartment of the chamber. Following 8-h incubation at 37°C with 5% CO₂, the cells were fixed with 100% methanol for 1 min at room temperature and stained with hematoxylin and eosin (3 min at room temperature). The non-migrated cells were removed by a cotton swab. Images of the migrated cells were captured in five fields by an Olympus CKX53 microscope (magnification, x10) and counted manually.

Cell viability assay. A549 cells (2×10^4 cells/well) treated with ginsenoside Rg5, and subsequently incubated at 37°C with 5% CO₂. After 24 h, cells were subjected to Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc.) according to the manufacturer's instructions (incubated at 37°C for 30 min). Absorbance at 450 nm was assessed using a microplate reader.

Statistical analysis. Data are presented as the mean \pm SD. Statistical analyses were performed using GraphPad Prism 7

software (GraphPad Software, Inc.). The differences between two groups were analyzed by Student's t-test. Differences among multiple groups were analyzed by one-way ANOVA with Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Ginsenoside Rg5 suppresses TNF- α -induced p65 activation. Ginsenosides were screened to identify compounds that exert inhibitory effects on p65 activation using HeLa cells stably transfected with a p65-dependent reporter plasmid (data not shown), and the results demonstrated that ginsenoside Rg5 (Fig. 1A) inhibited TNF- α -induced p65 activation compared with that in untreated cells (Fig. 1B). Following TNF- α stimulation, p65 is phosphorylated and transferred to the nucleus (3,5). As presented in Fig. 1C-E, the phosphorylation of p65 (Figs. 1C and D, and S1A and B) and its nuclear translocation (Figs. 1E and S1C) were inhibited by the ginsenoside Rg5 pretreatment. Ginsenoside Rg5 inhibited not only the TNF- α -, but also the IL-1 β -induced phosphorylation of p65 in A549 cells (Figs. 1F, S1D, S2 and S3). These results suggested that ginsenoside Rg5 suppressed the activation of p65 by inhibiting the phosphorylation of p65 and nuclear translocation of p65 independently of cell and cytokine types.

Ginsenoside Rg5 inhibits TAK1 phosphorylation. To elucidate the molecular mechanism by which ginsenoside Rg5 inhibits the activation of p65, the phosphorylation levels of the upstream molecules of p65, namely TAK1, IKK and I κ B α , were evaluated (Figs. 2A and S4A-C). TAK1 and IKK were phosphorylated 5 min post-TNF- α stimulation, whereas ginsenoside Rg5 moderately inhibited the phosphorylation of these kinases. A band shift in I κ B α , which indicated the phosphorylation of I κ B α , occurred after 5 min and completely degraded after 10 min; ginsenoside Rg5 inhibited the phosphorylation and degradation of I κ B α (Figs. 2A and S4A-C). Similar results were obtained in the TNF- α - or IL-1 β -treated A549 cells (Figs. S5A and B, and S6A and B). These results demonstrated that ginsenoside Rg5 inhibited the phosphorylation of TAK1 to suppress the activation of p65. TAK1 is stabilized by binding with its adaptor proteins TAB1 and TAB2; to investigate the potential interactions between ginsenoside Rg5 and the TAK1 complex, the present study used a cellular thermal shift assay, which identifies compound-protein interactions based on increased stability of the complex compared with that of a single protein at the expected denaturation temperature, resulting in higher protein expression levels in the complex-forming sample compared with those in the non-forming sample at a specific temperature (20). As presented in Figs. 2B and S4D-F, denaturation of TAK1 was observed at 46°C, and TAK1 was mostly denatured at 49°C. At 46-49°C, the expression levels of TAK1 were higher in ginsenoside Rg5-treated cells compared with those in untreated cells, suggesting that ginsenoside Rg5 bound to the TAK1 complex. The expression levels of TAB1 and TAB2 were also higher in ginsenoside Rg5-treated cells compared with those in untreated cells at 49°C and 43-46°C, respectively. Similar results were obtained for A549 cells (Figs. S5C and S6C-E) or TAK1-, TAB1- or TAB2-overexpressing 293T cells (Figs. S5D

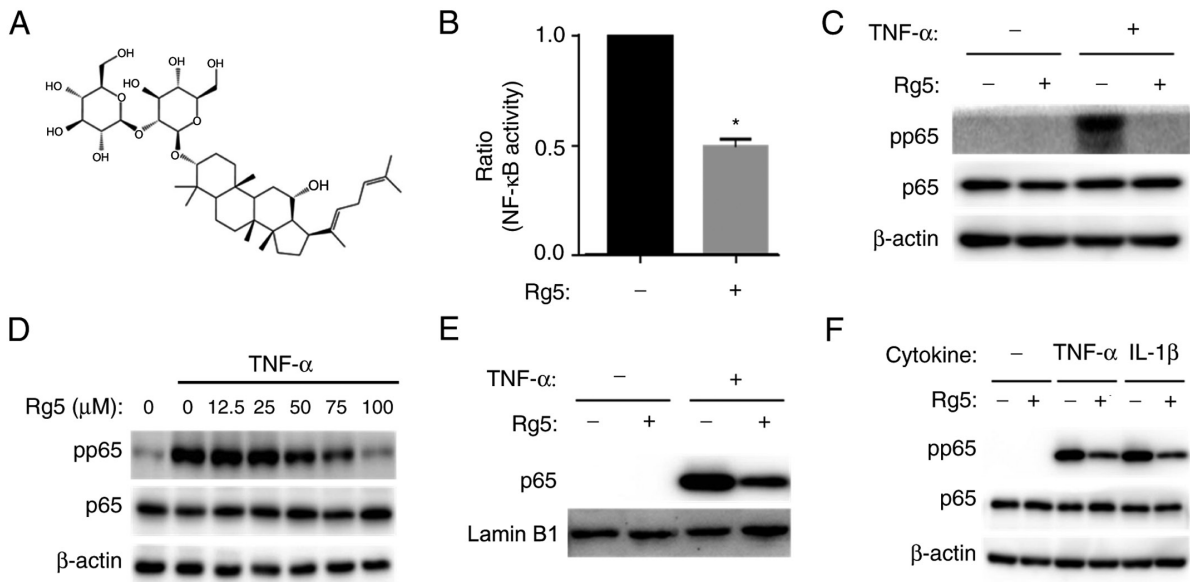


Figure 1. Inhibitory effects of ginsenoside Rg5 on cytokine-induced p65 activation. (A) The chemical structure of ginsenoside Rg5. (B) Luciferase activity of HeLa cells transfected with NF-κB luciferase reporter plasmids treated with DMSO or 50 μM ginsenoside Rg5 for 30 min and stimulated with TNF-α. The ratio represents NF-κB activity in the ginsenoside Rg5 pre-treated sample vs. control. n=3. *P<0.0001. (C and D) Western blotting analysis of p65 expression and phosphorylation levels in the lysates of HeLa cells treated with DMSO and (C) 50 μM or (D) the indicated concentrations of ginsenoside Rg5 for 30 min and stimulated with TNF-α. (E) Western blotting analysis of p65 expression and phosphorylation levels in the nuclear extracts of HeLa cells treated with DMSO or 50 μM ginsenoside Rg5 for 30 min and stimulated with TNF-α for 10 min. (F) Western blotting analysis of p65 expression and phosphorylation levels in A549 cells treated with DMSO or 50 μM ginsenoside Rg5 for 30 min and stimulated with TNF-α or IL-1β. pp65, phosphorylated p65.

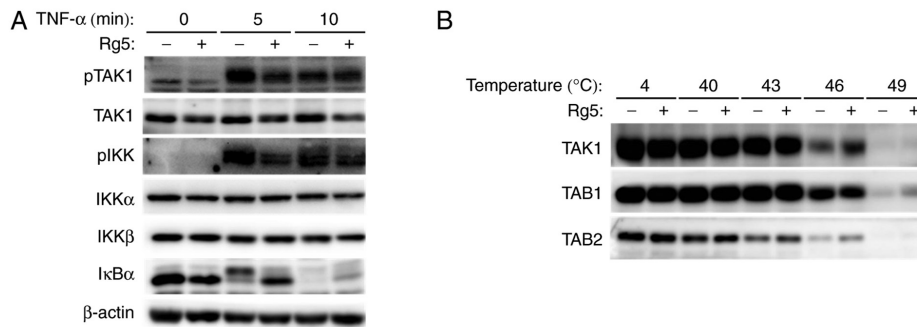


Figure 2. Molecular mechanisms by which ginsenoside Rg5 inhibits p65 activation. (A) Western blotting analysis of TAK1 and IKK expression levels in HeLa cells treated with DMSO or 50 μM ginsenoside Rg5 and exposed to TNF-α for the indicated time periods. (B) Thermal shift assay analysis of TAK1, TAB1 and TAB2 expression levels in HeLa cells were treated with DMSO or 50 μM ginsenoside Rg5. TAK1, transforming growth factor β-activated kinase 1; IKK, IκB kinase; TAB1, TAK1-binding protein 1; TAB2, TAK1-binding protein 2.

and E and S6F-H). Therefore, these results suggested that ginsenoside Rg5 directly interacted with the TAK1 complex to inhibit TAK1 phosphorylation and suppress its downstream NF-κB signaling.

Ginsenoside Rg5 inhibits the phosphorylation of EphA2 at S897. The inhibition of NF-κB signaling represents one strategy for suppressing cancer metastasis; however, our previous study demonstrated that TNF-α-induced activation of the RSK-EphA2 pathway promoted cell motility (8). Therefore, the effects of ginsenoside Rg5 on the RSK-EphA2 pathway were further investigated. As demonstrated in Figs. 3A and S7A, 50 μM ginsenoside Rg5 inhibited the levels of TNF-α-induced S897 phosphorylation compared with those in the DMSO-treated cells. However, the phosphorylation of RSK and its upstream kinase ERK was not suppressed by

ginsenoside Rg5. The expression levels of EphA2 were slightly suppressed by treatment with ginsenoside Rg5 compared with those in the DMSO group (Figs. 3A and S7B). These effects were also observed in TNF-α- and IL-1β-stimulated A549 cells (Figs. 3B, S7C-E, S8A and B and S9A-F). To elucidate the molecular mechanisms underlying the suppression of EphA2 by ginsenoside Rg5, the mRNA levels of EphA2 were determined in HeLa (Fig. S8C) and A549 (Fig. S8D) cells, and the results demonstrated that they were not reduced by ginsenoside Rg5 treatment compared with those in the DMSO-treated cells. A previous study reported that the EphA2 ligand-mediated tyrosine phosphorylation of EphA2 promoted EphA2 endocytosis to induce the proteasomal degradation of EphA2 (6). As demonstrated in Figs. S8E and F, and S9G and H, EphA2 phosphorylation at Tyr-588 was not induced by ginsenoside Rg5. Protein degradation is mediated through

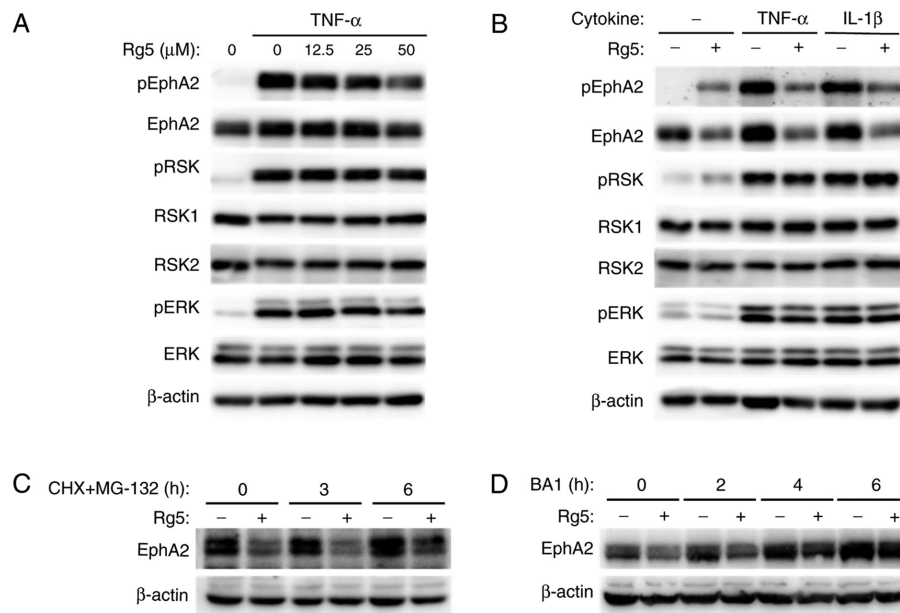


Figure 3. Effects of ginsenoside Rg5 on the RSK-EphA2 pathway. (A and B) Western blotting analysis of the expression and phosphorylation levels of proteins involved in the RSK-EphA2 pathway in (A) HeLa cells treated with various concentrations of ginsenoside Rg5 and stimulated with TNF- α , and (B) A549 cells treated with DMSO or 50 μ M ginsenoside Rg5 and exposed to TNF- α or IL-1 β . (C and D) Expression levels of EphA2 in HeLa cells (C) pre-treated with 50 μ g/ml CHX and 10 nM MG-132 or (D) 10 nM BA1 for the indicated times at 37°C and treated with DMSO or 50 μ M ginsenoside Rg5 for 30 min. Cell lysates were immunoblotted with anti-EphA2 and anti- β -actin antibodies. EphA2, erythropoietin-producing hepatocellular receptor A2; RSK, p90 ribosomal S6 kinase; CHX, cycloheximide; BA1, bafilomycin A1; p, phosphorylated.

the ubiquitin/proteasome or lysosomal system. To establish whether ginsenoside Rg5 reduces EphA2 protein expression by these two pathways, HeLa and A549 cells were pre-treated with the proteasome inhibitor MG-132 and protein synthesis inhibitor cycloheximide or a specific inhibitor of vacuolar-type H(+)-ATPase to block lysosomal trafficking bafilomycin A1. Co-treatment with MG-132 and cycloheximide did not inhibit the reduction of EphA2 expression levels in HeLa compared with that in the ginsenoside Rg5-treated cells (Figs. 3C and S7F) or A549 (Figs. S8G and S9I) cells, indicating that the suppression of EphA2 expression was not dependent on the ubiquitin/proteasome system. By contrast, following pre-treatment with bafilomycin A1 and treatment with ginsenoside Rg5 in HeLa (Figs. 3D and S7G) or A549 (Figs. S8H and S9J) cells, the reduction of EphA2 expression in ginsenoside Rg5-treated cells was gradually inhibited, and it was completely inhibited following 6 h pre-treatment. Therefore, these data suggested that ginsenoside Rg5 induced the degradation of EphA2 through the lysosomal system, and, as a result, ginsenoside Rg5 inhibited the phosphorylation of EphA2 at S897.

Ginsenoside Rg5 inhibits cell migration. The NF- κ B and RSK-EphA2 pathways have been reported to induce cell migration (3,5,8). We hypothesized that the activation of the NF- κ B and RSK-EphA2 pathways may exert additive effects on cell migration. To analyze this, p65-, RSK- and EphA2-expressing plasmids were transfected into 293 cells, and cell migration was detected by wound healing assay. The expression levels of p65, RSK1 and EphA2 are presented in Fig. S10A. As presented in Figs. 4A and S10B, cell migration appeared to be promoted in the p65- and EphA2+RSK1-transfected cells compared with that in the control group. In addition, migration was strongly promoted in both p65- and EphA2+RSK1 co-overexpressing

cells compared with that in the control, p65 and EphA2+RSK1 single transfection groups. To clarify whether NF- κ B and RSK-EphA2 signaling induced by inflammatory cytokines promoted cell migration, and whether ginsenoside Rg5 inhibited these effects, A549 cells were pre-treated with ginsenoside Rg5 and stimulated with TNF- α . TNF- α significantly promoted cell migration, whereas ginsenoside Rg5 pre-treatment suppressed TNF- α -induced cell migration without affecting cell viability (Figs. 4B and C, and S10C). Similarly, a migration assay using Transwell chambers revealed that ginsenoside Rg5 inhibited TNF- α - and IL-1 β - induced cell migration (Figs. 4D and S10D). These results demonstrated that the activation of p65 and the RSK-EphA2 signaling pathway exerted additive effects on cell migration, whereas ginsenoside Rg5 attenuated cell migration by inhibiting NF- κ B and EphA2 signaling.

Discussion

The results of the present study demonstrated that ginsenoside Rg5 inhibited p65 and EphA2 phosphorylation by suppressing TAK1 activity and EphA2 expression levels, respectively, compared with those in untreated cells. In addition, the NF- κ B and RSK-EphA2 pathways exerted additive effects on cell migration. To the best of our knowledge, the present study is the first study to directly demonstrate these effects, suggesting that the inhibition of these two pathways may be crucial for abolishing cancer migration. The present results suggest that ginsenoside Rg5 may be a powerful compound for inhibiting cancer migration.

Previous studies have reported that ginsenoside Rg5 inhibits the NF- κ B signaling pathway. Lee *et al* (17) have demonstrated that ginsenoside Rg5 exerts anti-inflammatory effects

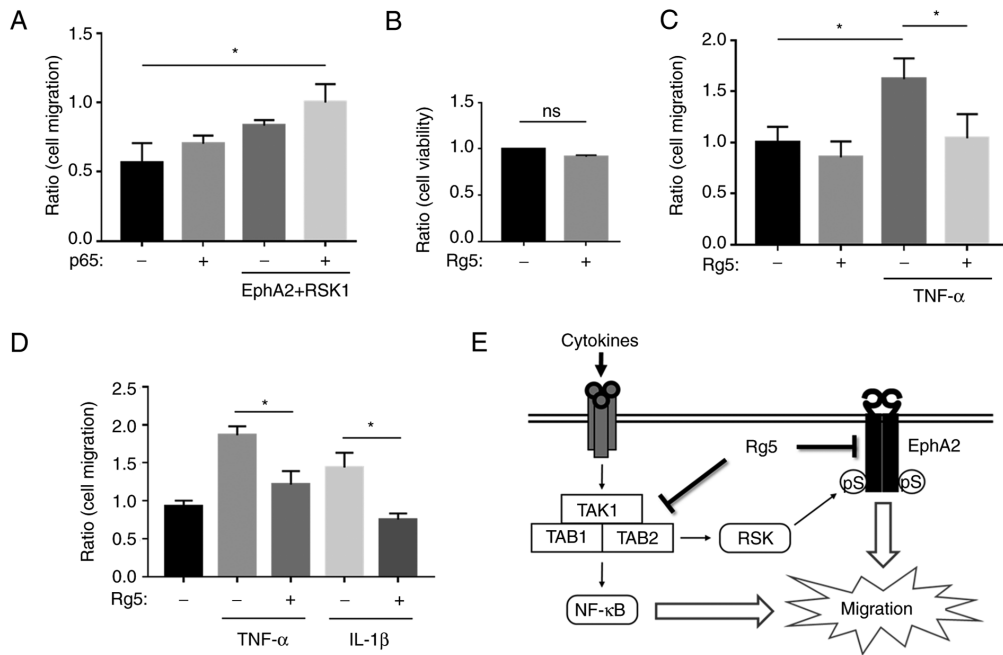


Figure 4. Ginsenoside Rg5 inhibits cell migration. (A) Migration of 293 cells transfected with p65, RSK1 and EphA2 expression plasmids determined by wound healing assay. The ratio of cell migration distance is indicated (vs. p65 + EphA2 + RSK1-transfected cells). (B) The viability of A549 were treated with DMSO or 50 μ M ginsenoside Rg5 measured by Cell Counting Kit-8. The ratio of cell viability is presented (vs. untreated cells). (C) Migration of A549 cells treated with DMSO or 50 μ M ginsenoside Rg5 followed by a TNF- α stimulation determined by wound healing assay. The ratio of cell migration distance is indicated (vs. DMSO-treated cells). (D) Transwell migration assay of A549 cells treated with DMSO or 50 μ M ginsenoside Rg5, followed by TNF- α or IL-1 β stimulation. Migrated cell numbers are presented as a ratio to the untreated control group. (E) Schematic diagram of the effects of ginsenoside Rg5 on NF- κ B and EphA2 signaling. ns, non-significant; *P<0.05. EphA2, erythropoietin-producing hepatocellular receptor A2; RSK, p90 ribosomal S6 kinase; TAK1, transforming growth factor β -activated kinase 1; TAB1, TAK1-binding protein 1; TAB2, TAK1-binding protein 2; pS, phosphorylation at S897.

by inhibiting the phosphorylation of NF- κ B in BV2 cells, and Kim *et al* (18) reported that ginsenoside Rg5 suppresses the phosphorylation of NF- κ B and translocation of p65 into the nucleus in breast cancer cells with inflammation. However, the molecular mechanisms by which ginsenoside Rg5 inhibits the NF- κ B pathway currently remain unclear. The results of the present study demonstrated that ginsenoside Rg5 inhibited NF- κ B signaling by suppressing the activation of TAK1. Furthermore, these results demonstrated that ginsenoside Rg5 directly bound to the TAK1 complex. TAK1 serves multiple functions in inflammation and is associated with a number of diseases, such as tumors and diabetes (4). The present study not only revealed the effects of ginsenoside Rg5 on the TAK1 complex, but also provided an important strategy for the development of TAK1 inhibitors; however, the structural properties of ginsenoside Rg5 binding to the TAK1 complex were not elucidated. Further studies are needed to determine the 3-dimensional structure of the TAK1/TAB1/TAB2 protein bound to ginsenoside Rg5 using X-ray crystallography to validate these results in order to develop specific TAK1 inhibitors for clinical applications.

Accumulating evidence has demonstrated the importance of EphA2 expression and phosphorylation at S897 in tumor malignancy, including tumor metastasis, the properties of cancer stem cells and antitumor drug resistance (6,7). Regarding the relationship between the NF- κ B and EphA2 pathways, Hong *et al* (22) have reported that the expression of EphA2 and its ligand Ephrin-A1 is induced in mice with lipopolysaccharide-induced lung injury, and an EphA2 monoclonal antibody inhibits the activation of NF- κ B as well as AKT, SRC proto-oncogene and ribosomal protein S6 kinase B1 in a mouse model. By contrast,

Funk *et al* (23) have suggested that Ephrin-A1 does not affect the activation of the NF- κ B signaling pathway in human aortic endothelial cells. The present study also attempted to clarify the potential crosstalk between the NF- κ B and EphA2 signaling pathways. In the cells used in the present study, TNF- α -induced NF- κ B phosphorylation was not affected by the knockdown of EphA2 (data not shown). Therefore, there appeared not to be any overlap between NF- κ B and EphA2 signaling in these cells. However, these experiments were limited and may not provide enough data to fully elucidate this. The signaling cascade is complex and exhibits different reactions in various cell types or stimuli; therefore, to determine the relationship between the NF- κ B and EphA2 signaling pathways, further evidence is needed, for instance, by using EphA2 and p65 knockout cell lines to analyze the two signaling cascades.

The results of the present study suggested that the induction of RSK phosphorylation was promoted by ginsenoside Rg5 (Figs. 3A and B). A previous study has reported that ginsenoside Rg5 induces the activation of insulin-like growth factor-1 receptor (IGF-1R) and promotes the phosphorylation of ERK in human umbilical vein endothelial cells (24). HeLa and A549 cells also express IGF-1R (25). Therefore, ginsenoside Rg5 may also induce the phosphorylation of ERK by activating IGF-1R in these cells. In A549 cells harboring a KRAS mutation, the basal level of ERK phosphorylation is high (26). Furthermore, in the present study, the induction of ERK phosphorylation by ginsenoside Rg5 was weak (data not shown); difficulties were associated with detecting the induction of ERK phosphorylation in A549 cells. Therefore, its induction in A549 cells treated with ginsenoside Rg5 was not

clear (Fig. 3B). In the present study, the induction of ERK phosphorylation following ginsenoside Rg5 treatment was detected in HeLa cells (data not shown). Therefore, ginsenoside Rg5 may induce the activation of IGF-1R and ERK, followed by the phosphorylation of its downstream kinase RSK.

To the best of our knowledge, the present study was the first to demonstrate that ginsenoside Rg5 suppressed the expression of EphA2 by promoting its degradation using the lysosomal system. Although other ginsenosides that have been demonstrated to exert anti-inflammatory effects were also tested in the present study (data not shown), only ginsenoside Rg5 promoted the degradation of EphA2. In addition, the expression of other receptor tyrosine kinases, such as epidermal growth factor receptor, was detected in ginsenoside Rg5-treated cells (data not shown), and the results revealed that their expression was not reduced, suggesting that ginsenoside Rg5 specifically induced the degradation of EphA2. Previous studies have reported that the ubiquitin/proteasome system degrades specific proteins; by contrast, the lysosomal system performs non-specific protein degradation (27,28). Therefore, further studies are needed to fully elucidate the molecular mechanisms of action of ginsenoside Rg5 on the lysosomal system.

Ginseng and its main components ginsenosides have been reported to exert antimetastatic effects; however, further evidence is needed to elucidate the underlying mechanisms. Although *in vivo* studies are required, the results of the present study provide a scientific basis for the potential development of ginsenoside Rg5 as an antimetastatic drug.

Acknowledgements

The authors would like to thank Professor Hiroaki Sakurai (University of Toyama, Toyama, Japan) for providing the transfection plasmids.

Funding

This work was supported by the National Natural Science Foundation of China (grant nos. 81603156 and 81920108033), the Young Eastern Scholar Program (grant no. QD2016038), the Chenguang Program (grant no. 16CG49), the Shanghai Sailing Program (grant no. 18YF1421700) and the MSD Life Science Foundation, Public Interest Incorporated Foundation.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding authors on reasonable request.

Authors' contributions

LS conducted the experiments and wrote the manuscript. ZW and LY designed the study. FY and YZ designed the study, conducted the experiments and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Mehlen P and Puisieux A: Metastasis: A question of life or death. *Nat Rev Cancer* 6: 449-458, 2006.
- Crusz SM and Balkwill FR: Inflammation and cancer: Advances and new agents. *Nat Rev Clin Oncol* 12: 584-596, 2015.
- Karin M: Nuclear factor-kappaB in cancer development and progression. *Nature* 441: 431-436, 2006.
- Sakurai H: Targeting of TAK1 in inflammatory disorders and cancer. *Trends Pharmacol Sci* 33: 522-530, 2012.
- Bharti AC and Aggarwal BB: Nuclear factor-kappa B and cancer: Its role in prevention and therapy. *Biochem Pharmacol* 64: 883-888, 2002.
- Zhou Y and Sakurai H: Emerging and diverse functions of the EphA2 noncanonical pathway in cancer progression. *Biol Pharm Bull* 40: 1616-1624, 2017.
- Wykosky J and Debinski W: The EphA2 receptor and ephrinA1 ligand in solid tumors: Function and therapeutic targeting. *Mol Cancer Res* 6: 1795-1806, 2008.
- Zhou Y, Yamada N, Tanaka T, Hori T, Yokoyama S, Hayakawa Y, Yano S, Fukuoka J, Koizumi K and Sakurai H: Crucial roles of RSK in cell motility by catalyzing serine phosphorylation of EphA2. *Nat Commun* 6: 7679, 2015.
- Song H and Lee YJ: Inhibition of hypoxia-induced cyclooxygenase-2 by Korean Red Ginseng is dependent on peroxisome proliferator-activated receptor gamma. *J Ginseng Res* 41: 240-246, 2017.
- Kee JY, Han YH, Mun JG, Park SH, Jeon HD and Hong SH: Effect of Korean Red Ginseng extract on colorectal lung metastasis through inhibiting the epithelial-mesenchymal transition via transforming growth factor- β 1/Smad-signaling-mediated Snail/E-cadherin expression. *J Ginseng Res* 43: 68-76, 2019.
- Poudyal D, Cui X, Le PM, Hofseth AB, Windust A, Nagarkatti M, Nagarkatti PS, Schetter AJ, Harris CC and Hofseth LJ: A key role of microRNA-29b for the suppression of colon cancer cell migration by American ginseng. *PLoS One* 8: e75034, 2013.
- Gao Q and Zheng J: Ginsenoside Rh2 inhibits prostate cancer cell growth through suppression of microRNA-4295 that activates CDKN1A. *Cell Prolif* 51: e12438, 2018.
- Jang KJ, Choi SH, Yu GJ, Hong SH, Chung YH, Kim CH, Yoon HM, Kim GY, Kim BW and Choi YH: Anti-inflammatory potential of total saponins derived from the roots of *Panax ginseng* in lipopolysaccharide-activated RAW 264.7 macrophages. *Exp Ther Med* 11: 1109-1115, 2016.
- Mao H, Jiang C, Xu L, Chen D, Liu H, Xu Y, Ma K and Wang M: Ginsenoside protects against AKI via activation of HIF-1 α and VEGF-A in the kidney-brain axis. *Int J Mol Med* 45: 939-946, 2020.
- Nguyen NH and Nguyen CT: Pharmacological effects of ginseng on infectious diseases. *Inflammopharmacology* 27: 871-883, 2019.
- Liang LD, He T, Du TW, Fan YG, Chen DS and Wang Y: Ginsenoside-Rg5 induces apoptosis and DNA damage in human cervical cancer cells. *Mol Med Rep* 11: 940-946, 2015.
- Lee YY, Park JS, Jung JS, Kim DH and Kim HS: Anti-inflammatory effect of ginsenoside Rg5 in lipopolysaccharide-stimulated BV2 microglial cells. *Int J Mol Sci* 14: 9820-9833, 2013.
- Kim SJ and Kim AK: Anti-breast cancer activity of Fine Black ginseng (*Panax ginseng* Meyer) and ginsenoside Rg5. *J Ginseng Res* 39: 125-134, 2015.
- Waiwut P, Shin MS, Inujima A, Zhou Y, Koizumi K, Saiki I and Sakurai H: Gomisins N enhances TNF- α -induced apoptosis via inhibition of the NF- κ B and EGFR survival pathways. *Mol Cell Biochem* 350: 169-175, 2011.
- Jafari R, Almqvist H, Axelsson H, Ignatushchenko M, Lundbäck T, Nordlund P and Martinez Molina D: The cellular thermal shift assay for evaluating drug target interactions in cells. *Nat Protoc* 9: 2100-2122, 2014.
- Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-Delta Delta} C(T) method. *Methods* 25: 402-408, 2001.

22. Hong JY, Shin MH, Douglas IS, Chung KS, Kim EY, Jung JY, Kang YA, Kim SK, Chang J, Kim YS and Park MS: Inhibition of EphA2/EphrinA1 signal attenuates lipopolysaccharide-induced lung injury. *Clin Sci (Lond)* 130: 1993-2003, 2016.
23. Funk SD, Finney AC, Yurdagul A Jr, Pattillo CB and Orr AW: EphA2 stimulates VCAM-1 expression through calcium-dependent NFAT1 activity. *Cell Signal* 49: 30-38, 2018.
24. Cho YL, Hur SM, Kim JY, Kim JH, Lee DK, Choe J, Won MH, Ha KS, Jeoung D, Han S, *et al*: Specific activation of insulin-like growth factor-1 receptor by ginsenoside Rg5 promotes angiogenesis and vasorelaxation. *J Biol Chem* 290: 467-477, 2015.
25. The Human Protein Atlas. Available from: <https://www.protein-atlas.org/>.
26. The Catalogue Of Somatic Mutations In Cancer (COSMIC): COSMIC v92, released 27-AUG-20. Available from: <https://cancer.sanger.ac.uk/cosmic>.
27. Kwon YT and Ciechanover A: The ubiquitin code in the ubiquitin-proteasome system and autophagy. *Trends Biochem Sci* 42: 873-886, 2017.
28. Ganley IG: Autophagosome maturation and lysosomal fusion. *Essays Biochem* 55: 65-78, 2013.