2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside suppresses human colorectal cancer cell metastasis through inhibiting NF-κB activation

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Received April 10, 2016; Accepted June 3, 2016

DOI: 10.3892/ijo.2016.3574

Abstract. 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside (THSG), a major component of *Polygonum multiflorum* Thunb (He-Shou-Wu), has been reported to exhibit antioxidant and anti-inflammatory effects. However, its anti-metastatic effect against colorectal cancer is still unclear. In this study, cell migration, invasion and adhesion abilities as well as metastasis-associated protein and NF-κB pathway signaling factor expression were analyzed after treating HT-29 cells with THSG. According to the results, the migration and invasiveness of HT-29 cells were reduced after treatment with 5 or 10 mM THSG ($p<0.05$). Additionally, the levels of matrix metalloproteinase-2 (MMP-2) and phosphorylated VE-cadherin in HT-29 cells were reduced and the transepithelial electrical resistance (TEER) of EA.hy926 endothelial cell monolayers was increased after incubation in THSG for 24 h ($p<0.05$). Cell adhesion ability and the E-selectin and intercellular adhesion molecule-1 (ICAM-1) protein levels were reduced when EA.hy926 endothelial cells were treated with THSG ($p<0.05$). In addition, the cytoplasmic phosphorylation of IκB, the nuclear p65 level and the DNA-binding activity of NF-κB were reduced after treating HT-29 or EA.hy926 cells with 5 or 10 mM THSG ($p<0.05$). These results suggest that THSG inhibits HT-29 cell metastasis by suppressing cell migration, invasion and adhesion. Furthermore, THSG inhibits metastasis-associated protein expression by suppressing NF-κB pathway activation.

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Abbreviations: THSG, 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside; MMP, matrix metalloproteinase; TEER, transepithelial electrical resistance; ICAM-1, intercellular adhesion molecule-1; ECM, extracellular matrix; VE, vascular endothelial; BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; BM, basement membrane; EMT, epithelial-mesenchymal transition

Key words: 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside, migration, invasion, adhesion, metastasis, NF-κB, human colorectal cancer cells

Introduction

Colorectal cancer (CRC) is the third most lethal malignancy in the world, and the major cause of death of CRC patients is metastasis (1). Furthermore, it is known that the potential for a tumor cell to metastasize depends on its interactions with the homeostatic factors that promote tumor-cell growth, survival, angiogenesis, invasion and metastasis (2). During tumor metastasis, two key events are invasion and metastatic colonization. Both of these events occur because of interactions between the cancer cells and the stromal microenvironment. Tumor cell invasion of the surrounding extracellular matrix (ECM), which involves migration and intravasation, is the early stage of metastasis (3). It is known that ECM degradation is an important stage of tumor metastasis that is regulated by matrix metalloproteinases (MMPs) (4). Among the MMP family members, MMP-2 and MMP-9 contribute to the degradation of the ECM and play important roles in cancer cell migration and invasion (5). In addition, vascular endothelial (VE)-cadherin is an important molecule that modulates cell-cell and cell-ECM contact permeability and extravasation (6). Moreover, when cancer cells leave the original tumor organ and enter the blood or lymphatic circulation via intravasation, the cells will migrate to and invade a metastatic target organ, which is referred to as extravasation. During this process, adhesion molecules (e.g., E-selectin and ICAM-1) play a key role in regulating the adhesion of tumor cells to endothelial cells (2,7). Inhibiting cell migration, invasion and adhesion may be an effective strategy for improving the prognosis of CRC.

The transcription factor NF-κB plays an important role in cell metastasis and invasion signaling pathways (8). Several genes that are known to be involved in tumor metastasis, such as the genes encoding MMP-2, MMP-9, E-selectin, ICAM-1, and VE-cadherin, are regulated by NF-κB. All of these factors are downstream targets of the NF-κB signaling pathway. When NF-κB is abnormally overexpressed, these metastasis-relevant genes may be activated, leading to the initiation of cancer cell metastasis.

2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucopyranoside (THSG) is one of the most abundant components of *Polygonum multiflorum* Thunb (He-Shou-Wu), a traditional Chinese medicine and medicinal food component (9). Many physiological studies demonstrated that THSG has various protective effects against acute and chronic colitis, neurodegenerative disorders and atherosclerosis (10-12). However, cellular studies to investigate the effects of THSG on anticancer and antimetastasis are still limited. THSG belongs to stilbenoids with a hydroxystilbene structure (Fig. 1) (9). The resveratrol, piceatannol and pterstilbene types of stilbenoids can inhibit metastasis through suppressing migration and invasion *in vivo* and *in vitro* (10-12). These stilbenoids all are hydroxylated derivatives of stilbene and have a C6-C2-C6 structure. Additionally, THSG has a specific glycoside structure (9). A previous study showed that THSG has antitumor and antimetastatic activity via inhibiting DNA synthesis in Lewis lung carcinoma tumors and human umbilical vein endothelial cells (13). Since THSG is biologically active, more cellular studies to clarify the potential effects of THSG on anticancer and anti-metastasis are worth further investigation.

The aims of this study were to determine whether THSG exerts anti-metastatic effects and whether this effect on human CRC cells involves the regulation of cell migration, invasion or adhesion. Wounding healing assays, cell invasion assays, and measurements of the activity and protein expression of MMPs such as MMP-2 and MMP-9 were analyzed in previous studies to examine the migration and invasion ability (14-17); these techniques were also employed in this study. In addition, cell adhesion capacity, the levels of adhesion molecules such as ICAM-1 and E-selectin, and transepithelial electrical resistance (TEER) were measured to evaluate cell adhesion and invasion abilities in this study. In addition, whether THSG regulates the expression of metastasis-associated molecules including MMP-2, MMP-9, E-selectin and ICAM by altering NF-κB activation was analyzed in this study. These experiments helped to elucidate how THSG regulates the migration and invasion of human CRC cells and the molecular signal transduction pathways involved in this process.

Materials and methods

Chemicals and reagents. THSG (HPLC purity 95%) was purchased from Zhongxin Pharmaceuticals (Tianjin, China). Antisera against E-selectin, ICAM-1 phosphorylated (p-) VE-cadherin, p-IκB and NF-κB were purchased from Abcam (Cambridge, MA, USA). Antisera against MMP-2 and MMP-9 were obtained from GeneTex, Inc. (San Antonio, TX, USA). 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) was purchased from Life Technologies GmbH (Darmstadt, Germany).

Cell cultures and treatment. In this study, the HT-29 human colorectal carcinoma cell line was used as a model of CRC metastasis (18), and EA.hy926 human endothelial cells were used as an experimental model of invasion and adhesion (19). HT-29 cells and EA.hy926 cells were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). The HT-29 and EA.hy926 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2.

In our pre-tests, various THSG concentrations ranging from 10 μM to 50 mM were applied for 24 or 48 h for cell viability analysis. The pre-test results showed that 1, 5 or 10 mM THSG treatment significantly decreased the levels of both MMP-2 and E-cadherin in HT-29 cells without reducing cell viability (data not shown). Thus, 1, 5 and 10 mM THSG were predominantly used in our further experiments. After plating and incubating the cells for 24 h, the cells were treated with THSG diluted in dimethyl sulfoxide (DMSO) for various biochemical analyses. Cells treated with DMSO alone were used as a control group.

Cell viability analysis. Cell viability was evaluated using the 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay as described by Debizot and Lang (20). HT-29 cells were incubated in 3-cm plates ($1x10^6$ cells) for 24 h and treated with 1, 5, 10 or 50 mM THSG for 24, 48 or 72 h. Then, MTT (5 mg/ml) dye was added, and the optical density (OD) was measured at 570 nm.

Wound healing assay. To investigate the effects of THSG on the migration ability of human colorectal carcinoma cells, a wound healing assay was conducted. The protocol for the wound-healing assay was a modified version of a protocol described by Ang *et al* (21). HT-29 cells were cultured on 3-cm plates ($1x10^6$ cells) for 24 h, and a micropipette tip was used to make a uniform scratch in the center of the well. The cells were then washed with PBS. Various concentrations of THSG (1, 5 or 10 mM) were added to the respective wells for the indicated duration (24, 48 or 72 h), and the morphology of HT-29 cells was observed under an inverted fluorescence microscope (Olympus IX51 Microscope, Olympus Optical Co. Ltd., Tokyo, Japan). The width of the wound area was measured using ImageJ software to determine the cell migration distance. The wound closure rate for a given treatment period was calculated as follows: % wound area closure = (wound area of control group-wound area of treatment group)/wound area of control group x 100. The migration ability of HT-29 cells is suppressed when the wound closure rate is lower.

Invasion assay. The invasion assay is a method for assessing intravasation and extravasation abilities. A Matrigel solution (50 μl) was added to the wells of a 24-well Transwell plate that was incubated at 37˚C for 30 min. Next, HT-29 cells were resuspended in serum-free RPMI-1640 medium ($5x10^4$ cells/3-cm plate) in the absence or presence of THSG (1, 5 or 10 mM) in the upper chamber. RPMI-1640 medium (500 μl) containing 10% FBS was added to the lower chamber. After incubation for 24 h, the invading cells that had migrated to the lower surface of the filter membrane were stained with 0.2% crystal violet

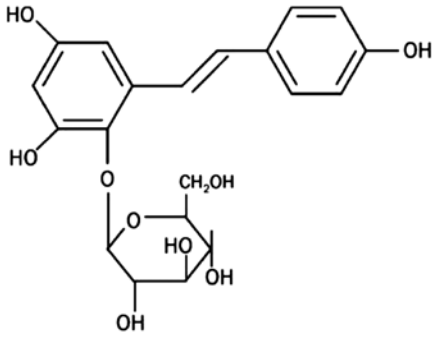

Figure 1. The structure of 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside (THSG).

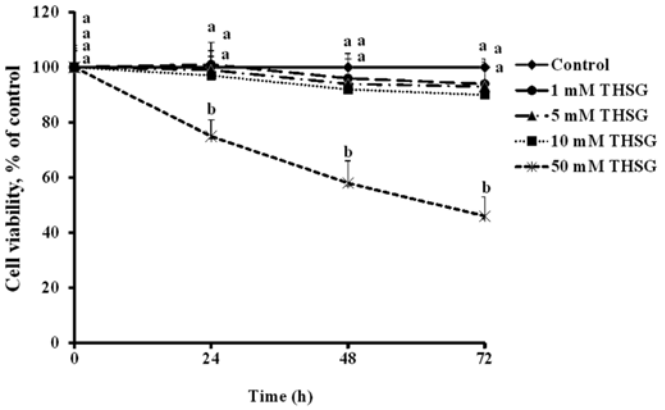

Figure 2. Effects of THSG on the viability of HT-29 cells. HT-29 cells ($5x10^5$ cells/ml) were treated with 0, 1, 5, 10 or 50 mM α-PA for 24 h. The DMSO treatment group served as the control. The values are presented as the means ± SD (n=3). [a,b]Groups in the same treatment period with different letters are significantly different according to Duncan's test ($p<0.05$).

for 15 min. The number of invading cells on the lower surface of the filter membrane was counted under an inverted fluorescence microscope (Olympus IX51 Microscope, Olympus Optical Co. Ltd.) using NIH ImageJ software (22).

TEER measurement. TEER is a quantitative measurement of the barrier integrity of a monolayer (23). For the TEER measurement, EA.hy926 cells ($5x10^4$ cells/well) were cultured for 24 h in RPMI-1640 medium containing 1, 5 or 10 mM THSG. The TEER values were obtained by subtracting the TEER of the cell culture dish groove from the TEER in the presence of a cell layer. These measurements were recorded using a Millicell-ERS voltohmmeter (Millipore Continental Water Systems, Bedford, MA, USA).

Adhesion assay. The adhesion assay protocol was a modified version of a protocol described by Braut-Boucher *et al* (24). EA.hy926 cells ($1x10^6$ cells) were cultured on 3-cm plates and exposed to 1, 5 or 10 mM THSG for 6 h. The EA.hy926 cells were then washed in PBS and co-cultured with HT-29 cells labeled with 10 μM BCECF for 1 h. After washing in PBS, the morphology of BCECF-stained HT-29 cells was evaluated under an inverted fluorescence microscope (Olympus IX51 Microscope, Olympus Optical Co. Ltd.). Furthermore, the BCECF-stained cells were collected and measured fluorometrically using an ELISA reader at an OD of 580 nm.

Analysis of the expression of proteins regulating cell migration, adhesion, and invasion and NF-κB activation. Approximately $5x10^5$ HT-29 cells/3-cm plate, which were used for the MMP-2, MMP-9, p-VE-cadherin, p-IκB, IκB, and cytoplasmic and nuclear NF-κB expression analyses or $5x10^5$ EA.hy926 cells/3-cm plate, which were used for the E-selectin, ICAM-1, p-IκB, and cytoplasmic and nuclear NF-κB expression analyses, were incubated in a 12-well plate in the presence of 0, 1, 5 or 10 mM THSG for 24 h.

The cells were washed twice with cold PBS and then harvested in 200 μl of lysis buffer containing 10 mM Tris-HCl, 5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 20 μg/ml aprotinin at pH 7.4. The cellular protein levels were determined using the method described by Lowry *et al* (25). For each sample, 10-20 mg of cellular proteins were applied to 10% sodium dodecyl sulfate (SDS) polyacrylamide gels (26). After electrophoresis, the proteins that had separated on the gels were transferred to polyvinylidene difluoride membranes (27). The membranes were then incubated with anti-MMP-2, anti-MMP-9, anti-E-selectin, anti-ICAM-1, anti-p-VE-cadherin, anti-p-IκB, anti-IκB or anti-NF-κB antibodies at 37˚C for 1 h, followed by incubation with a peroxidase-conjugated secondary antibody. The bands were visualized using hydrogen peroxide/diaminobenzidine tetrahydrochloride or an enhanced chemiluminescence detection kit (Amersham Life Science, Buckinghamshire, UK); then, the band densities were quantified using an AlphaImager 2000 imaging system (Alpha Innotech, San Leandro, CA, USA). The protein level of the control group was regarded as 100%.

NF-κB DNA-binding activity assay. Nuclear extracts were obtained from cell pellets using an NE-PER extraction kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. The NF-κB DNA-binding activity of the nuclear fraction was determined using an NF-κB (p65) transcription factor activity assay kit (Cayman Chemical Co., Ann Arbor, MI, USA) according to the manufacturer's instuctions.

Statistical analysis. The data were analyzed using the statistical analysis software SPSS for Windows, version 20.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) and Duncan's multiple range tests were used to evaluate the significance of the differences between two mean values. A p-value <0.05 was considered to be statistically significant.

Results

THSG suppresses the migration of human colorectal carcinoma cells. The cell viability of HT-29 cells treated with 1, 5 or 10 mM THSG did not significantly differ from the control cells during the 72-h incubation period. However, the viability of the cells treated with 50 mM THSG for 24, 48 or 72 h was significantly lower than that of the controls ($p<0.05$) (Fig. 2).

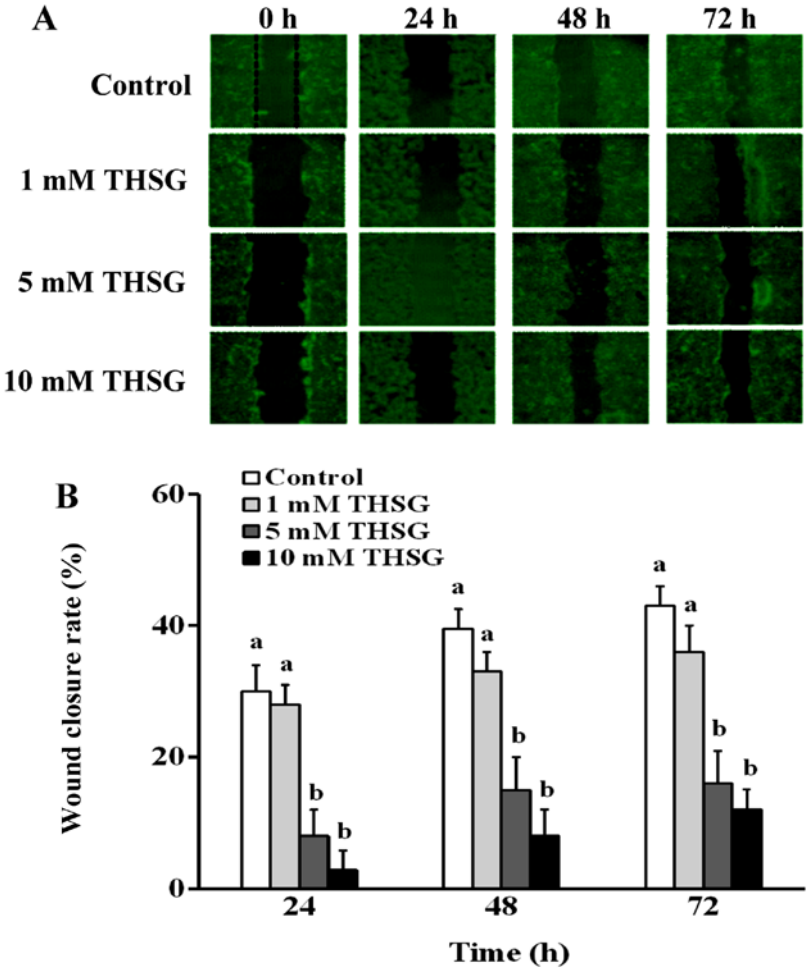

Figure 3. Effects of THSG on the migration of HT-29 cells. HT-29 cells ($5x10^5$ cells/3-cm plate) were treated with 1, 5 or 10 mM THSG for 24, 48 or 72 h. The DMSO treatment group served as the control. (A) The effect of THSG on HT-29 cell migration *ex vivo*. The movement of HT-29 cells into the wound was demonstrated using a wound healing assay. (B) Quantitative analysis of the wound closure rate. The values are presented as the means ± SD (n=3). [a,b]Groups in the same cell phase with different letters are significantly different according to Duncan's test ($p<0.05$).

To determine whether the migration of HT-29 cells is suppressed by THSG, a monolayer wound healing assay was conducted. As shown in Fig. 3A, after the HT-29 cells were incubated in various concentrations of THSG for 24, 48 or 72 h, the wound healing areas varied. When HT-29 cells were treated with 5 or 10 mM THSG for 24, 48 or 72 h, their migration was inhibited by 22-27 and 27-31%, respectively (Fig. 3B). These results indicated that THSG significantly decreased HT-29 cell migration.

THSG reduces the invasiveness of human colorectal carcinoma cells and their passage across human endothelial cells. The results of a versatile Transwell Matrigel invasion assay indicated that when HT-29 cells were incubated in various concentrations of THSG for 24 h, the percentages of invading cells were lower than those of the control cells (Fig. 4A and B). The relative abundances of invading HT-29 cells were 84±12, 47±17 and 32±15% of the control levels after treatment for 24 h with 1, 5 and 10 mM THSG, respectively. Cell invasion was significantly suppressed in all of the THSG-treated groups compared with the control group (100%) ($p<0.05$). This result shows that THSG may reduce inter-colon cell migration and invasion by HT-29 cells.

The effect of THSG on the TEER of EA.hy926 cells was also analyzed. As shown in Fig. 4C, treatment of EA.hy926 cells with 5 or 10 mM THSG significantly increased the TEER values to 194±40 and 213±37% of the control levels (100%), respectively ($p<0.05$). This result shows that THSG may increase the inter-epithelial cell TEER, thus decreasing EA.hy926 cell-cell permeability, intravasation, and extravasation.

THSG regulates the adhesion of human colorectal carcinoma cells to human endothelial cells. The fluorescence microscopy data shown in Fig. 5A indicate that treatment of EA.hy926 cells with various concentrations of THSG reduced the number of adherent cells in a dose-dependent manner after co-culture with HT-29 cells. As shown in Fig. 5B, the percentages of EA.hy926 cells that adhered to HT-29 cells were 96±3, 88±7 and 68±5% for the 1, 5, and 10 mM treatment groups, respectively, relative to the control group (100%). Percentages of adherent cells in the 5 and 10 mM THSG groups were significantly lower than that in the control group (100%) after the 24-h incubation period ($p<0.05$). These results demonstrate that THSG can decrease the ability of HT-29 cells to adhere to EA.hy926 cells, thereby reducing cancer cell adhesion to a metastatic target organ.

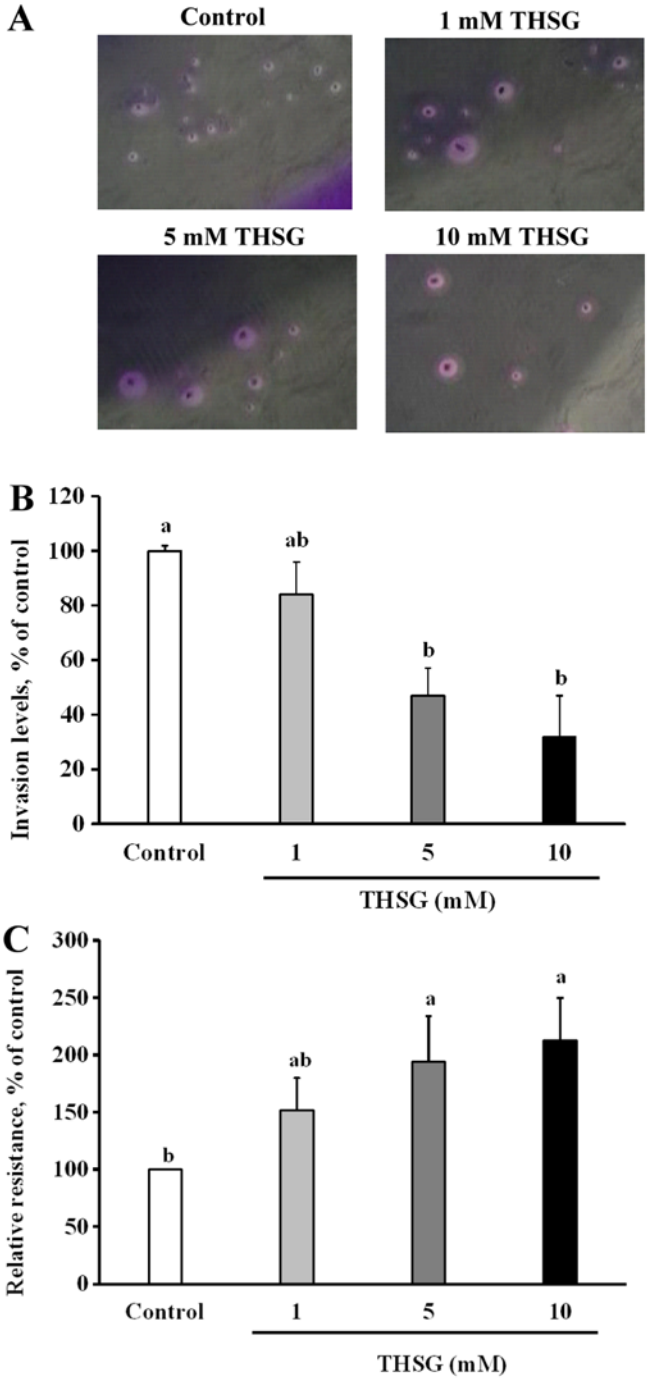

Figure 4. Effect of THSG on cell invasion and transepithelial electrical resistance. HT-29 cells or EA.hy926 cells ($5x10^5$ cells/3-cm Transwell plate) were treated with 1, 5 or 10 mM THSG for 24 h. The DMSO treatment group served as the control. (A) Transwell invasion assay showed that the THSG inhibited cell invasion of HT-29 cells. Images were captured at a magnification of x200. Representative of three independent experiments. (B) Quantitative analysis of HT-29 cell invasion across the Matrigel membrane, from the upper chamber to the lower chamber. (C) The effect of THSG on transepithelial electrical resistance (TEER) in EA.hy926 cells. The values are presented as the means ± SD (n=3). a or b groups with different letters significantly differ from each other, and ab groups are not significantly different from either a groups or b group by Duncan's test ($p<0.05$).

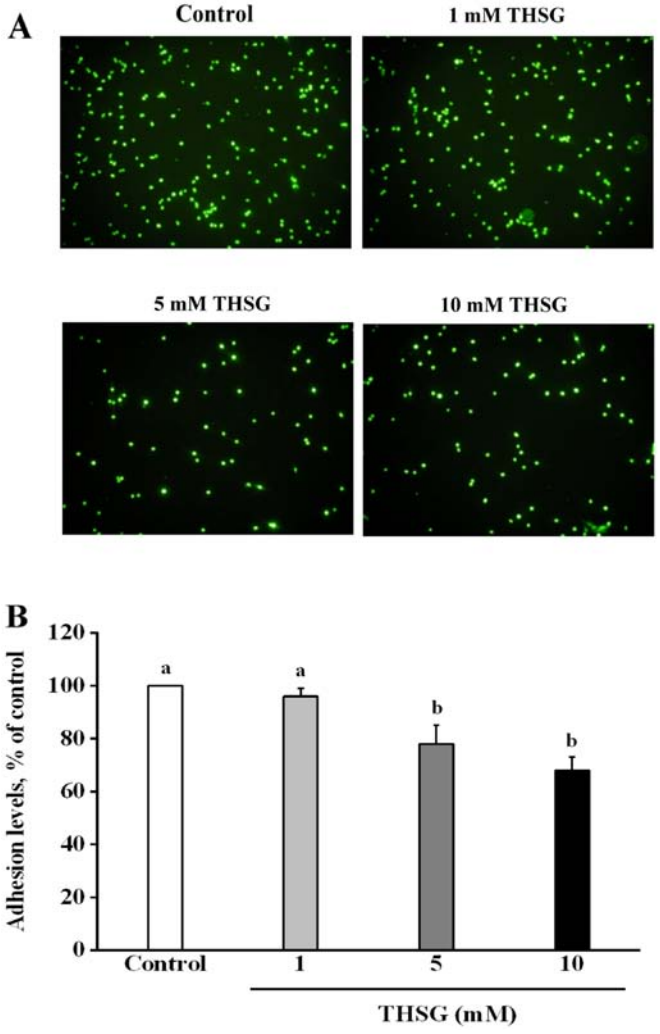

Figure 5. Effect of THSG on the adhesion of HT-29 cells. EA.hy926 cells ($1x10^6$ cells/3-cm plate) were treated with 0, 1, 5 or 10 mM THSG for 24 h. Then, HT-29 and EA.hy926 cells were co-treated for 1 h. (A) After the HT-29 cells were stained with BCECF, the adhesion of HT-29 cells to EA.hy926 cells was observed under a fluorescence microscope. (B) HT-29 cell staining was observed using a fluorescence ELISA reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The values are presented as the means ± SD (n=3). [a,b]Groups in the same cell phase with different letters are significantly different according to Duncan's test ($p<0.05$).

THSG suppresses the levels of metastasis-associated regulatory proteins. To elucidate the effects of THSG on the metastasis-related processes of migration, adhesion and invasion by HT-29 cells, we analyzed the expression of the proteins involved in these events. As shown in Fig. 6, HT-29 cells treated with 5 or 10 mM THSG for 24 h displayed MMP-2 protein levels that were significantly reduced to 38±6 and 25±8% of the control levels (100%), respectively ($p<0.05$). However, after treatment of HT-29 cells with various concentrations of THSG for 24 h, the MMP-9 protein level was not significantly altered compared with the control treatment. Fig. 6 also shows the levels of p-VE-cadherin, an important protein in cell-cell adherens junctions, following treatment with 5 or 10 mM THSG. The levels of p-VE-cadherin in the 5 and 10 mM THSG treatment groups were significantly reduced to 35±14 and 42±16% of the control values (100%), respectively. These results indicate that THSG can regulate cell migration and intravasation by suppressing MMP-2 expression and VE-cadherin phosphorylation.

With respect to cell adhesion, the protein levels of E-selectin and ICAM-1, both of which act as adhesion mole-

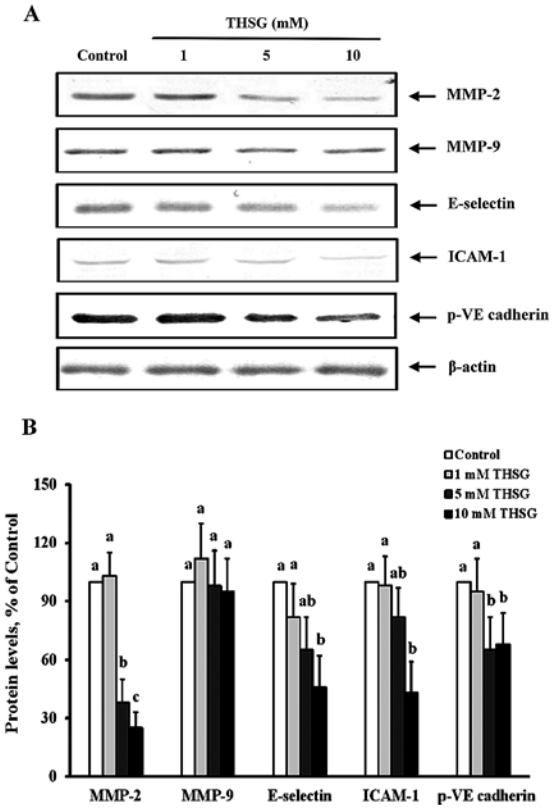

Figure 6. The expression levels of metastasis-regulating proteins following THSG treatment. HT-29 or EA.hy926 cells ($5x10^5$ cells/3-cm plate) were treated with 1, 5 or 10 mM THSG for 24 h. The DMSO treatment group served as the control. Immunoblotting assays were used to analyze the levels of MMP-2, MMP-9 and p-VE-cadherin in HT-29 cells. The E-selectin and ICAM-1 levels in EA.hy926 cells were also analyzed. The values were determined based on three independent experiments and are presented as the means ± SD. [a-c]Groups in the same cell phase with different letters are significantly different according to Duncan's test ($p<0.05$).

cules in EA.hy926 cells, were significantly reduced to 62±14 and 43±13% of the control levels (100%), respectively, in the 10 mM THSG treatment group ($p<0.05$) (Fig. 6). These results indicate that the expression of these adhesion molecules was suppressed by THSG and that this compound may perform an important anti-metastatic function.

THSG reduces NF-κB activation in HT-29 cells and EA.hy926 cells. In this study, to examine whether the regulation of MMP-2, MMP-9, E-selectin and ICAM-1 expression by THSG is dependent on the inhibition of NF-κB activation, both immunoblotting assays for an NF-κB regulatory molecule and NF-κB-DNA binding activity assays were performed. Fig. 7 shows the effects of THSG on NF-κB activation in HT-29 cells and EA.hy926 cells. According to the immunoblotting results, the levels of p-IκB and nuclear NF-κB were significantly reduced after treatment of HT-29 cells with 5 or 10 mM THSG for 24 h compared with the control treatment (100%) ($p<0.05$) (Fig. 7A and B). This result indicates that 5 and 10 mM THSG can reduce the translocation of NF-κB from the cytoplasm to the nucleus in HT-29 cells. Fig. 7C shows that the NF-κB-DNA binding activity of the HT-29 cells treated with 5 or 10 μM THSG was significantly reduced to approximately 14-22% of the control levels (100%) ($p<0.05$).

Similar results were observed in the EA.hy926 cells. Treatment of EA.hy926 cells with 5 or 10 mM THSG for 24 h significantly inhibited the phosphorylation of IκB and reduced the nuclear NF-κB level compared with the control treatment (100%) ($p<0.05$) (Fig. 7D and E). The NF-κB-DNA binding activity in the EA.hy926 cells was also inhibited by the 5 and 10 mM THSG treatments (22-32%) compared with the control group (100%) ($p<0.05$) (Fig. 7F). These results indicate that THSG can suppress the activation of NF-κB and regulate the protein expression of its downstream targets in HT-29 cells and EA.hy926 cells.

Discussion

In this study, THSG was shown to significantly suppress HT-29 cell migration, invasion and adhesion (Figs. 3-5) and to significantly regulate the expression of metastasis-associated proteins by suppressing the activation of NF-κB (Figs. 6 and 7). These findings are novel in terms of the anti-metastatic effects of THSG. Specifically, THSG is a hydroxylated derivative of stilbene that is an important stilbenoid compound. In terms of the anti-metastatic activities of stilbenoids, both resveratrol and its metabolite piceatannol were shown to significantly suppress cell migration and invasion by reducing wound healing and invasion abilities and MMP-2 and MMP-9 expression via the inhibition of PI3K3/Akt/NF-κB and AP-1 signaling in brain, prostate, breast, and pancreatic cancer cells (14-16,28). Additionally, pterostilbene can decrease cell migration and invasion by suppressing PI3K/NF-κB-mediated MMP-2 and MMP-9 expression in oral squamous cell carcinoma and hepatocellular carcinoma cells (16). In this study, our results showed that THSG treatment significantly decreased wound healing and invasion abilities and MMP-2 expression in HT-29 cells. Moreover, THSG significantly suppressed the ability of cancer cells to adhere to endothelial cells and reduced the levels of adhesion molecules (including ICAM-1 and E-selectin) and cell-cell permeability (based on TEER). This study provides data demonstrating that THSG inhibits cell migration, invasion, and adhesion, and these findings indicate that THSG can inhibit metastasis by reducing HT-29 cell adhesion to endothelial cells and cell-cell permeability.

Notably, resveratrol, piceatannol and pterostilbene possess a C6-C2-C6 structure. The optimal concentration of these three stilbenes for inhibiting cell migration and invasion is ~5-80 μM. However, THSG possesses an additional glycoside moiety (Fig. 1). Glycoside residues usually play an important role in the pharmacokinetic parameters of natural products. However, glycosylation increases the hydrophilicity of aglycone and promote its excretion via urine (13). Previous studies showed that 300 μM-24.6 mM THSG can prevent cardiotoxicity and upregulate melanin synthesis in various types of mouse cardiomyocytes and B16F1 melanoma cells (17,29). In this study, THSG exhibited an effective dose of 5-10 mM for anti-metastatic activity.

Some stilbenoids regulate MMP-2 or MMP-9 expression and others inhibit cell invasive ability; however, the molecular regulatory mechanism underlying these effects remains unclear. In this study, THSG not only suppressed cell migration and invasion but also inhibited molecular signaling by proteins involved in metastasis and NF-κB pathway activa-

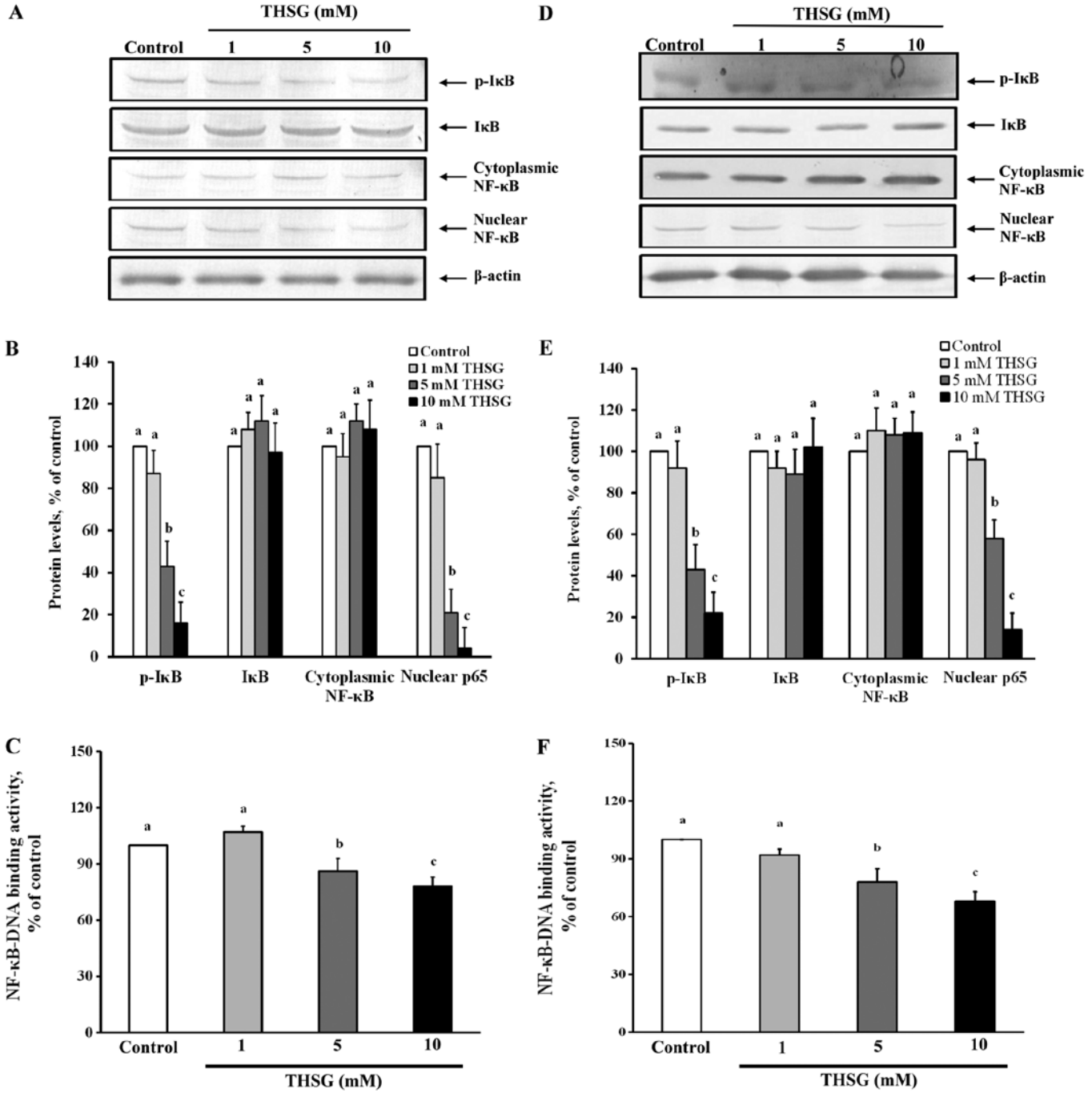

Figure 7. Level of NF-κB activation after THSG treatment in HT-29 and EA.hy926 cells. HT-29 and EA.hy926 cells ($5x10^5$ cells/3-cm plate) were treated with 1, 5 or 10 mM THSG for 24 h. The DMSO treatment group served as the control. Equal amounts of cytosolic proteins were separated and identified by SDS-PAGE followed by immunoblotting as described in Materials and methods. (A and D) Immunoblotting assays were used to analyze the levels of p-IκB, IκB, cytoplasmic NF-κB and nuclear NF-κB in HT-29 or EA.hy926 cells. (B and E) Protein levels in HT-29 and EA.hy926 cells were quantified by densitometry, and the levels in the control group were considered as 100%. (C and F) DNA-binding activity was investigated in HT-29 and EA.hy926 cells. The values are presented as the means ± SD (n=3-4). $^{a-c}$Groups with different letters are significantly different for a given target protein ($p<0.05$) (ANOVA and Duncan's multiple comparison test).

tion. Nearly 50% of all CRC patients develop colorectal liver metastasis, which is the main cause of death among patients with CRC (1). Therefore, in addition to research on targeted anti-metastasis therapy, the search for a herb or phytochemical to treat metastatic colon cancer is a new direction of research. THSG could potentially be used to prevent CRC metastasis.

When tumor cells undergo invasion and migration, they alter their surrounding ECM and basement membrane (BM) proteins to create a route for cell migration, and this process is regulated by MMPs (4). It is well known that MMP inhibitors block endothelial cell activities that are essential for cell proliferation and invasion (30). A previous study showed that fucoidan exerts a concentration-dependent inhibitory effect on the invasion and migration of A549 human lung cancer cells by decreasing the activity of MMP-2 (31). Gefitinib was found to inhibit MMP-9 and MMP-2 secretion and mRNA expres-

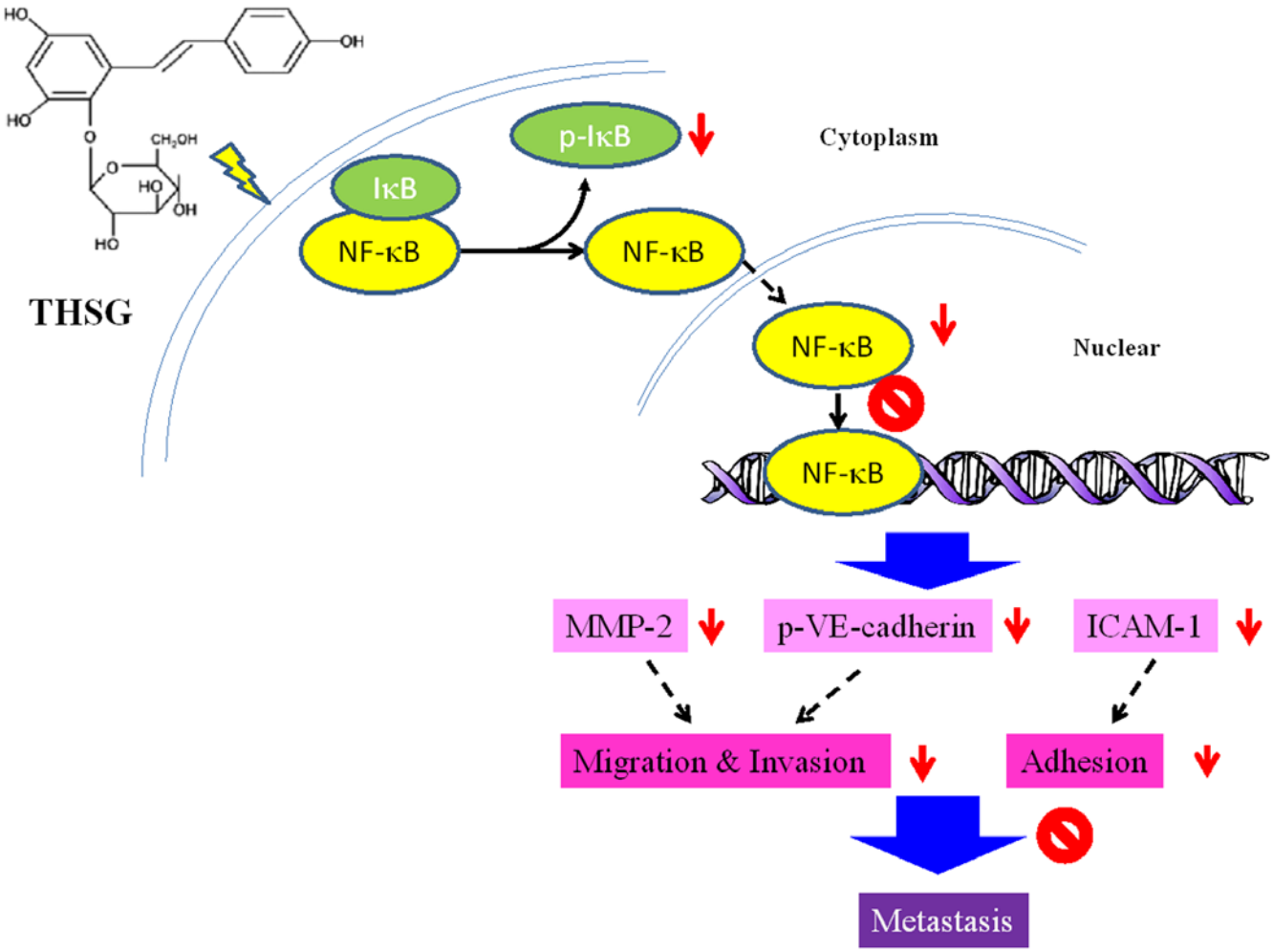

Figure 8. Possible mechanisms by which THSG inhibits human colorectal cancer cell metastasis.

sion, suppressing the metastasis of colon cancer HT-29 cells (32). Gallic acid suppressed PC-3 human prostate cancer cell migration and invasion by inhibiting the MMP-2 and MMP-9 signaling pathways (33). Our results showed that HT-29 cells treated with 5 or 10 mM THSG exhibited significantly reduced MMP-2 protein levels (Fig. 6). The suppressive effect of THSG on MMP-2 expression may be an important contributor to its anti-migratory effect.

Regarding tumor metastasis, tumor cells degrade the ECM via the regulation of MMP expression as well as other mechanisms, thereby allowing the cells to break away from the primary tumor. In the metastatic process, primary tumor cells undergo epithelial-mesenchymal transition (EMT), which involves morphogenetic changes that enhance cell motility and plasticity, allowing the cells to migrate to the circulation to become circulating tumor cells (34). Then, these circulating tumor cells roll along the endothelial cell surface, on which E-selectin is expressed (35). This event allows tumor cells to transmigrate from the endothelium to a metastatic site (36). In addition, ICAM-1 plays a critical role in regulating metastasis. Inhibiting ICAM-1 has been shown to block the invasion of lung cancer cells *in vitro* (37). A previous study showed that cannabidiol elicited a concentration-dependent increase in ICAM-1 expression, inhibiting the adhesion and extravasation of A549, H358, and H460 lung cancer cells (38). When cancer cells leave the original tumor organ, they intravasate into the circulatory system to migrate to the metastatic target organ. Adhesion molecules (e.g., E-selectin and ICAM-1) play a key role in regulating tumor adhesion, which is involved in this process (2). The E-selectin-mediated adhesion of tumor cells to the vascular endothelium was shown to be crucial in the progression of extravasation and metastasis in previous studies (39). In this study, we found that 10 mM THSG significantly inhibited the expression of both E-selectin and ICAM-1 (Fig. 6). Therefore, THSG can reduce HT-29 cell adhesion by decreasing the E-selectin and ICAM-1 levels.

Additionally, our results demonstrated that THSG dose-dependently reduced endothelial permeability, as indicated by the TEER values (Fig. 5), and suppressed the phosphorylation of VE-cadherin (Fig. 6) in HT-29 cells. These results provide compelling evidence that THSG can suppress the extravasation of HT-29 cells. It is known that cadherins are transmembrane proteins with various important functions during cell-cell adhesion in normal and cancerous cells (40). Among the cadherin family members, VE-cadherin plays an important role in endothelial cell adherens junctions and the regulation of vascular permeability, invasion and angiogenesis during tumor progression and metastasis. Endothelial permeability- and junction-related proteins play important roles in extravasation (41). VE-cadherin is a strictly endothelial-

specific adhesion molecule located at the junctions between endothelial cells. These junctions are associated with cell-cell adhesion structures and enable intercellular communication. The phosphorylation of VE-cadherin inactivates this protein and increases cell monolayer permeability, promoting tumor invasiveness (42). Moreover, endothelial cell-cell permeability can be monitored via the TEER, which potentially serves as an indicator of cancer cell extravasation (43). The expression of MMPs and junction proteins is important for cell membrane permeability and tumor cell extravasation (44). A previous study demonstrated that *Capsosiphon fulvescens*, a green alga, significantly suppresses cell invasion by decreasing MMP-2 and MMP-9 expression and increasing TEER in human gastric cancer AGS cells (45). THSG also inhibits MMP-2 expression and increases TEER in HT-29 cells, suppressing extravasation and metastasis. Our results show that THSG, which is the active principle component of *Polygonum multiflorum*, exerts anti-migratory effects by suppressing the expression of MMP-2. This compound also performs anti-invasion functions by inhibiting the expression of VE-cadherin and reducing epithelial permeability by elevating TEER. Moreover, THSG displays anti-adhesion properties that are mediated by the inhibition of E-selectin and ICAM-1 protein expression.

In this study, THSG was significantly affected by the expression of specific cell migration-, invasion- and adhesion-associated genes, e.g., MMP-2, E-selectin and ICAM, via the regulation of NF-κB activation, resulting in decreased protein levels of MMP-2, E-selectin and ICAM in HT-29 and EA.hy926 cells (Fig. 6). NF-κB is sequestered in the cytoplasm by its interaction with the inhibitory protein IκB. Exposure to a variety of external stimuli causes the phosphorylation of IκBα, which leads to the disassociation of NF-κB from IκBα (46). Activated NF-κB then translocates to the nucleus, where it binds to the cis-acting κB enhancer element of target genes such as MMP-2, MMP-9, E-selectin and ICAM and activates their transcription (47-49). Previous studies have shown that inhibiting NF-κB activity in human prostate cancer cells decreases their metastatic abilities by suppressing cell invasion via the downregulation of MMPs (50). As shown in Fig. 7, NF-κB pathway activation is downregulated in response to THSG treatment. These results indicate that NF-κB is an important cellular target of THSG for metastasis-associated gene regulation.

In conclusion, as shown in Fig. 8, our results support a model in which THSG suppresses CRC cell metastasis. THSG significantly altered the expression of specific migration-related genes, e.g., MMP-2 and ICAM-1, via the downregulation NF-κB activation, resulting in inhibited migration, invasion and adhesion by HT-29 and EA.hy926 cells. Thus, THSG may reduce the metastatic ability of colon cancer cells. In HT-29 cells, the mechanisms of action of THSG may involve the inhibition of cell migration, adhesion, intravasation and extravasation, as well as the regulation of protein expression. THSG might serve as a potential anti-metastatic agent.

Acknowledgements

This study was supported by E-Da Hospital (EDAHT102009) in Kaohsiung, Taiwan.

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