



## Research article

# Effect of Korean Red Ginseng extract on colorectal lung metastasis through inhibiting the epithelial–mesenchymal transition via transforming growth factor- $\beta$ 1/Smad-signaling-mediated Snail/E-cadherin expression



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## ABSTRACT

**Background:** In colorectal cancer (CRC), 40–60% of patients develop metastasis. The epithelial–mesenchymal transition (EMT) is a pivotal and intricate process that increases the metastatic potential of CRC. The aim of this study was to investigate the effect of Korean Red Ginseng extract (RGE) on colorectal metastasis through inhibition of EMT and the metastatic abilities of CRC cells.

**Methods:** To investigate the effect of RGE on the metastatic phenotypes of CRC cells, CT26 and HT29 cells were evaluated by using an adhesion assay, a wound-healing assay, an invasion assay, zymography, and real-time reverse transcription–polymerase chain reaction. Western-blot analysis was conducted to elucidate the molecular mechanisms of RGE, which showed an inhibitory effect on the transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1)-induced EMT in HT29 cells. Additionally, the antimetastatic effect of RGE was evaluated in a mouse model of lung metastasis injected with CT26 cells.

**Results:** RGE decreased the adhesion and migration ability of the CT26 cells and TGF- $\beta$ 1-treated HT29 cells. The invasion ability was also reduced by RGE treatment through the inhibition of matrix metalloproteinase-9 expression and activity. Moreover, RGE suppressed the TGF- $\beta$ 1-induced EMT via TGF- $\beta$ 1/Smad-signaling-mediated Snail/E-cadherin expression in HT29 cells and lung tissue in CT26 tumor-bearing mice.

**Conclusion:** Our results demonstrated that RGE inhibited colorectal lung metastasis through a reduction in metastatic phenotypes, such as migration, invasion, and the EMT of CRC cells.

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## 1. Introduction

Colorectal cancer (CRC) is the third most common malignant cancer worldwide. In the United States, 136,830 cases were newly diagnosed in 2014 [1]. Additionally, the incidence rate of CRC increased from 20% to 37% in Korea over a 13-yr period (1999–2011) [2]. Although the present surgical treatments and chemotherapy have improved the prognosis and survival rates of patients with CRC, metastasis remains the main cause of CRC-related mortality. The 5-yr survival rate is 90.3% for patients with localized tumors and only 12.5% for patients with distant metastases [1].

The epithelial–mesenchymal transition (EMT) is an essential morphogenetic process in tumor metastasis. During the EMT process, cells lose their epithelial properties, including cell–cell contact and cell polarity, and obtain mesenchymal properties, such as enhanced motility and invasion ability. The expression levels of EMT-related genes regulate the morphological changes from epithelial to mesenchymal, such as the spindle shape. The expression of the epithelial markers during the EMT process, including E-cadherin, is downregulated, whereas the mesenchymal markers, such as N-cadherin and Snail, are increased via Smad- or non-Smad-signaling pathways [3].

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Among the compounds that induce EMT, transforming growth factor- $\beta$  (TGF- $\beta$ ) promotes angiogenesis, migration, and invasion in a variety of cancer cells. In patients with CRC, a significant increase in TGF- $\beta$  was observed in the serum, and patients with high TGF- $\beta$  expression in the primary tumor suffered cancer recurrence [4,5]. Thus, the regulation of the TGF- $\beta$ -induced EMT is important for the improvement of CRC treatment and prognosis. TGF- $\beta$  activates the transcription factors Smad2/3, which form a complex with Smad4 and translocate to the nucleus to modulate the transcription of target genes. TGF- $\beta$  also controls Smad-independent pathways, such as the mitogen-activated-protein-kinase (MAPK)-signaling pathways, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase, and p38 [6].

Korean Red Ginseng is the heat-processed root of *Panax ginseng* Meyer. Korean Red Ginseng is a valuable and popular traditional medicinal herb in East Asia, where it is widely used to treat various diseases. The material is reported to have several pharmacological effects, including immunomodulatory, antifatigue, anti-allergic, and antitumor activities [7–10]. Ginsenosides Rg3, Rh1, Rh2, Rh4, Rs1–4, and Rf2 are known to be present only in Korean Red Ginseng, and these compounds exert pharmacological effects [11]. Ginsenoside Rh1 improved nonalcoholic fatty liver disease and chronic inflammatory disease [12,13], and ginsenoside Rh2 suppressed the proliferation of HCT116 human CRC cells and HepG2 hepatocarcinoma cells [14,15].

Notably, Korean Red Ginseng extract (RGE) and its active compound ginsenoside Rg3 exhibit antimetastatic effects. The extract suppressed the lung metastasis of CT26 cells and the metastatic potential of human hepatoma SK-Hep1 cells via a reduction in matrix metalloproteinase (MMP)-2/-9 activity [16,17]. Additionally, ginsenoside Rg3 decreased the lung metastasis of B16BL6 melanoma cells and azoxymethane plus bombesin-induced peritoneal metastasis of intestinal adenocarcinoma [18,19]. The present study was conducted to elucidate the effect of Korean Red Ginseng water extract on exacerbated colorectal lung metastasis by the EMT of CRC cells and related mechanisms.

## 2. Materials and methods

### 2.1. Reagents and antibodies

Recombinant human TGF- $\beta$ 1 was purchased from ProSpec (East Brunswick, NJ, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and RPMI 1640 were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Bouin's solution, Giemsa solution, and 4',6-diamidino-2-phenylindole were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against phospho-smad2/3, phospho-p38, phospho-ERK, smad2/3, p38, ERK, Snail, and E-cadherin were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

### 2.2. Preparation of RGE

RGE was manufactured and kindly provided by the Korea Ginseng Corporation (Daejeon, Korea). The extract was produced from the roots of a 6-yr-old ginseng plant (*P. ginseng* Meyer) harvested in Korea. The fresh ginseng was steamed at 90–100°C for 3 h, and then dried at 50–80°C. RGE was extracted three times by circulation in 85–90°C water for 8 h. The water content of the pooled extract was 36% of the total weight. The analysis of RGE by HPLC revealed the following contents of the major ginsenosides: Rb1, 7.44 mg/g; Rb2, 2.59 mg/g; Rc, 3.04 mg/g; Rd, 0.91 mg/g; Re,

1.86 mg/g; Rf, 1.24 mg/g; Rg1, 1.79 mg/g; Rg2, 1.24 mg/g; Rg3, 1.39 mg/g; and other minor ginsenosides.

### 2.3. Mice

Five-week-old BALB/c mice (male, 20–21 g) were obtained from Samtaco Korea (Osan, Korea). The animals were housed under controlled conditions (22  $\pm$  1°C and 55  $\pm$  1% humidity) in a laminar-airflow room. The research was performed in accordance with internationally accepted principles of the Animal Care and Use Committee of Wonkwang University (WKU-16-80).

### 2.4. Cell culture

The murine CRC cell line CT26, human CRC cell line HT29, human normal colon cell line CCD-18Co, and rat basophilic leukemia mast cell line RBL-2H3 were obtained from Korean Cell Line Bank (Seoul, Korea). HT29 cells were cultured in RPMI 1640, and CT26, CCD-18Co, and RBL-2H3 cells were maintained in DMEM supplemented with 10% FBS and penicillin–streptomycin (100 U/mL) in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

### 2.5. Cell viability

CT26 cells (3  $\times$  10<sup>3</sup> cells/well) and HT29, CCD-18Co, RBL-2H3 cells (1  $\times$  10<sup>4</sup> cells/well) were seeded in 96-well microplates. After incubation for 24 h with various concentrations of RGE, water-soluble tetrazolium (WST)-8 reagent (Enzo Life Sciences, Farmingdale, NY, USA) was added in the fresh medium. The colorimetric changes in absorbance were measured at 450 nm by a microplate reader.

### 2.6. Western blotting

Harvested cells and tissues were lysed for 30 min in lysis buffer (iNtRON Biotechnology, Seoul, Korea). After the cell lysates were centrifuged, the protein concentration was measured by the bicinchoninic-acid assay, and equal amounts of protein were mixed with a 2 $\times$  sample buffer. The total proteins were heated for 5 min at 95°C, separated by electrophoresis, and transferred to polyvinylidene-difluoride membranes. The membrane was blocked with 5% bovine serum albumin for 1 h and 30 min, and the primary antibodies were added to membranes to detect the specific proteins. After at least 3 h, the membranes were incubated with horseradish-peroxidase-conjugated secondary antibodies for 1 h. The secondary antibodies were detected using an enhanced-chemiluminescence solution.

### 2.7. Immunofluorescence

For immunofluorescence staining, cells (5  $\times$  10<sup>3</sup> cells/well) were seeded in an eight-chambered slide, and allowed to adhere overnight. After treatment with TGF- $\beta$ 1 and RGE, the cells were fixed with 3.7% formaldehyde, washed in phosphate-buffered saline (PBS) for 5 min, incubated with 0.1% Triton X-100/PBS for 10 min, and then blocked with a blocking buffer (3% bovine serum albumin, 0.3% Triton X-100 in PBS) for 1 h. After aspiration of the blocking buffer, the cells were incubated with primary antibodies overnight. The signals of the Alexa 488- and Alexa 568-conjugated secondary antibodies (Thermo Fisher Scientific) were observed and photographed using a Zeiss Observer A1 microscope (Carl Zeiss, Oberkochen, Germany).

## 2.8. Adhesion assay

Cells ( $5 \times 10^4$  cells/well) were pretreated with RGE for 24 h, and seeded in a Matrigel-coated 96-well plate for 4 h. The unattached cells were removed by washing, and the attached cells were fixed with 3.7% formaldehyde. After another wash with PBS, the cells were stained with 0.05% crystal-violet solution for 10 min. Randomly chosen fields were observed and images were obtained using an EVOS microscope (Thermo Fisher Scientific) at 200 $\times$  magnification.

## 2.9. Wound-healing and invasion assays

For the wound-healing assay, the cells were plated in six-well plates at  $1 \times 10^6$  cells, and cultured until 80–90% confluent. The confluent monolayer was wounded with a 200- $\mu$ L pipette tip, and the unattached cells were removed. The scratches were observed at 0 h and 24 h after incubation of the monolayers in the FBS-free medium plus the indicated concentrations of RGE. The invasion assay was conducted by using a Matrigel-coated Transwell chamber with 8.0-mm pore size. The upper part of the chamber was filled with cells ( $2.5 \times 10^4$  cells/250  $\mu$ L), and the lower part of the chamber was filled with DMEM plus 10% FBS. After incubation with RGE for 72 h, the attached cells were fixed with 3.7% formaldehyde and permeabilized by 100% methanol. The inner side of the chamber was stained with Giemsa solution, and the images were photographed under an EVOS phase-contrast microscope (Thermo Fisher Scientific).

## 2.10. Gelatin zymography

Gelatin zymography was conducted using a Zymogram buffer kit (KOMA BIOTECH, Seoul, Korea). The cells were incubated in six-

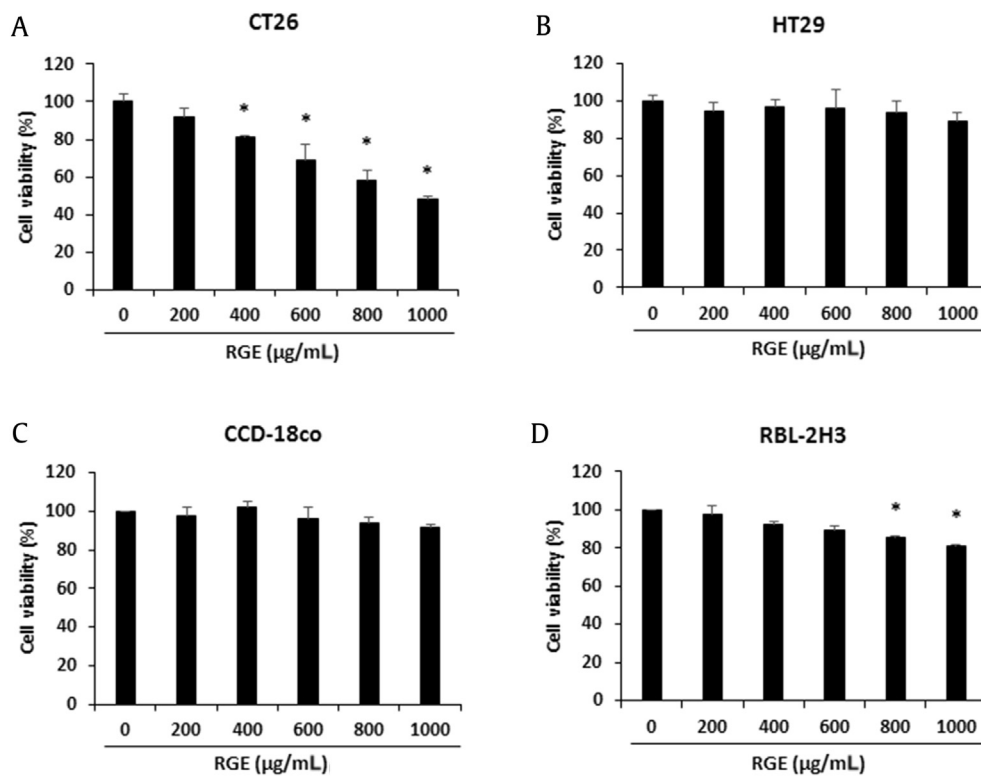
well plates ( $5 \times 10^5$  cells/well), and treated with RGE for 24 h. A sample of the conditioned medium was collected and mixed with 2 $\times$  sample buffer. MMP-9 was separated by electrophoresis in 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel containing 1% gelatin. The gels were washed in a renaturing buffer and incubated in a developing buffer at 37°C for 24 h. To determine the level of the MMP activity, the gels were stained with Coomassie Blue R-250 solution and destained with a destaining buffer (40% methanol and 10% acetic acid in distilled water).

## 2.11. Real-time reverse transcription–polymerase chain reaction

RNA-spin Total RNA Extraction Kit (iNtRON Biotechnology) was used to extract RNA in accordance with the recommended protocol. cDNA was synthesized from total RNA using a high-capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA), and amplified by StepOnePlus Real-Time PCR System (Applied Biosystems). The sequences of the primers used for the mouse genes were mmp-9, 5'-AGACCAAGGTTACAGCCTGTTC-3' and 5'-GGCAGCTGGAATGATCTAAG-3'; and GAPDH, 5'-GACATGCCGCTGGAGAAAC-3' and 5'-AGCCCAGGATGCCCTTGTAGT-3'. The sequences of the primers used for human genes were MMP-9, 5'-GAACCAATCTCACCGACAGG-3' and 5'-GAAACCCGAGTGTAACCATA-3'; and  $\beta$ -actin, 5'-AGAGCTACGAGCTGCCTGAC-3' and 5'-CGTGATGCCACAGGACT-3'. All data were normalized to GAPDH or  $\beta$ -actin mRNA levels.

## 2.12. Lung-metastasis mice model

CT26 cells were harvested and intravenously inoculated ( $2 \times 10^5$  cells/200  $\mu$ L/mouse) using an insulin syringe. The mice were sacrificed 2 wk after the injection, and lung tissue was fixed with Bouin's solution. The inhibitory effect of RGE on colorectal lung



**Fig. 1.** Cell viability of Korean Red Ginseng extract (RGE)-treated colorectal cancer cells and normal cells. (A) CT26, (B) HT29, (C) CCD-18Co, and (D) RBL-2H3 cells were seeded in a 96-well microplate, and treated with RGE (0–1,000  $\mu$ g/mL) for 24 h. The effects of RGE on cell proliferation were measured by WST assay. The results are expressed as the mean  $\pm$  standard deviation of three independent experiments. \* $p < 0.05$ . TGF- $\beta$ , transforming growth factor- $\beta$ .

metastasis was evaluated through the counting of metastatic nodules on the lung surface.

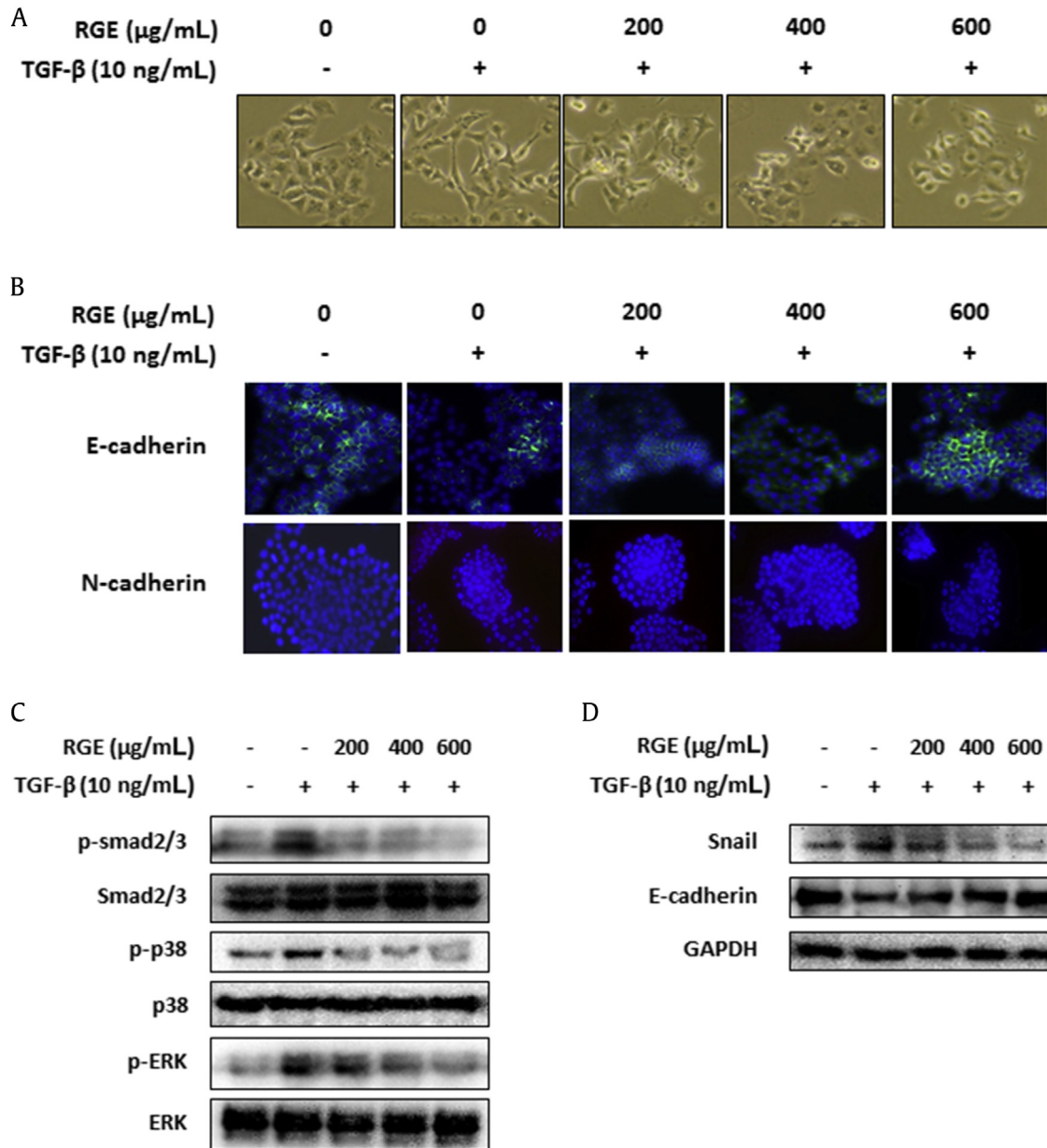
2.13. Statistical analysis

All statistical analyses were performed by Student *t* test using SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, USA). The results are presented as the mean ± standard deviation of three independent experiments. Values of *p* < 0.05 were considered to be statistically significant.

3. Results

3.1. Effects of RGE on the cell viability of CRC cells and normal colon cells

Various concentrations of RGE (0–1,000 µg/mL) were administered to CT26 and HT29 cells to evaluate the effect of RGE on the viability of CRC cells using WST reagent. As shown in Fig. 1A and 1B, high concentrations of RGE (400–1,000 µg/mL) reduced the viability of CT26 cells, whereas the viability of HT29 was unaffected.



**Fig. 2.** Korean Red Ginseng extract (RGE) suppressed epithelial–mesenchymal transition through the expression of Snail and E-cadherin via the Smad- and mitogen-activated protein kinase signaling pathways. (A) Effects of RGE on morphological changes of transforming growth factor-β1 (TGF-β1)-induced epithelial–mesenchymal transition in HT29 cells. The cells were treated with TGF-β1 (10 ng/mL) and RGE (200–600 µg/mL) for 72 h. (B) The expression of E-cadherin and N-cadherin was detected by immunofluorescence in (C) HT29 cells. The cells were plated in an eight-chambered slide and treated with various concentrations of RGE in the presence or absence of TGF-β1 for 72 h, respectively. (C) and (D) The Smad2/3- and p38/ERK-signaling pathways in TGF-β1-treated HT29 cells. TGF-β1 and RGE (200–600 µg/mL) were treated to cells for 72 h. (C) The phosphorylation of Smad2/3, p38, and ERK, and (D) the protein expressions of Snail and E-cadherin were confirmed by Western blotting. ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

To elucidate whether the RGE-induced antiproliferative effect was specific to CT26 cells, we confirmed the viability of normal colon cells and mast cells after RGE treatment. RGE did not decrease the viability of CCD-18Co cells (Fig. 1C); however, 800 and 1,000  $\mu\text{g}/\text{mL}$  RGE inhibited the proliferation of RBL-2H3, murine-derived mast cells (Fig. 1D). From these results, 200–600  $\mu\text{g}/\text{mL}$  RGE was selected for the experiments to evaluate the inhibitory effect on EMT and the metastatic phenotypes of HT29 cells.

### 3.2. Effects of RGE on the EMT and related mechanisms in CRC cells

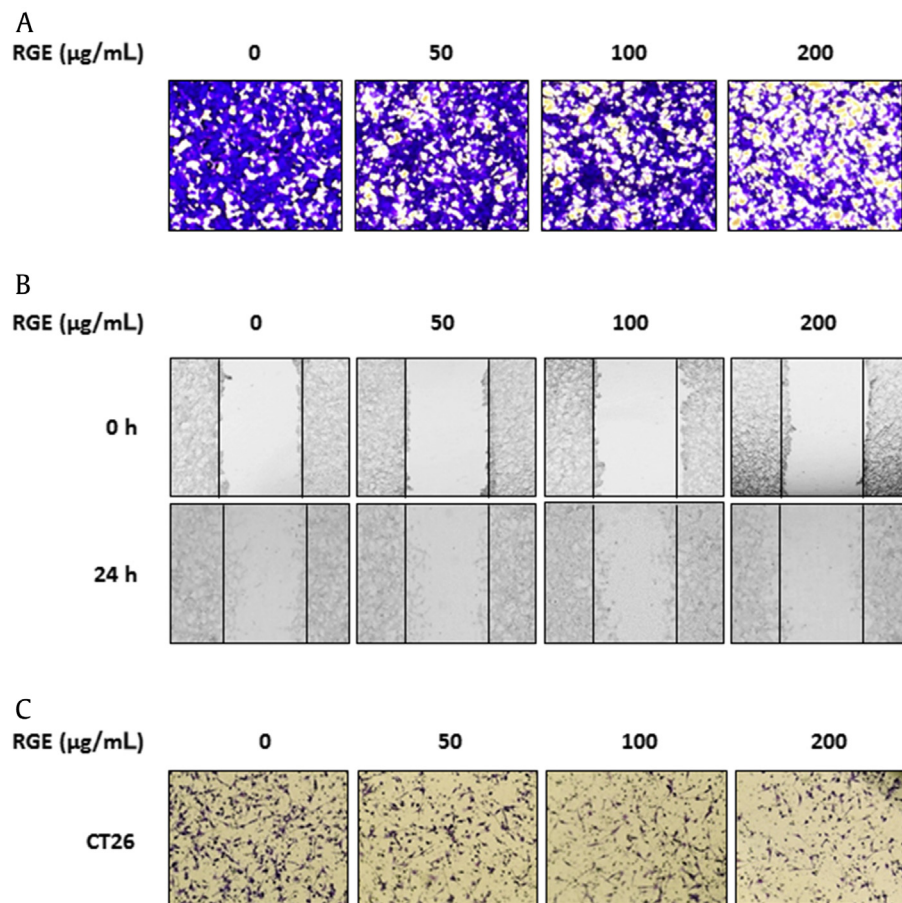
To investigate the effect of RGE on TGF- $\beta$ 1-induced EMT, the morphology of cells was observed under a microscope. As shown in Fig. 2A, treatment with TGF- $\beta$ 1 changed the morphology of HT29 cells to the mesenchymal phenotype, whereas the untreated cells displayed the epithelial phenotype. The treatment with RGE reduced the typical mesenchymal features, such as spindle-like and elongated shapes. The effect of RGE on the expression of E-cadherin and N-cadherin was determined in the CT26 and TGF- $\beta$ 1-treated HT29 cells by immunofluorescence staining, which confirmed the suppressive role of RGE during the EMT process. The expression of E-cadherin was increased, whereas N-cadherin expression was decreased in CT26 cells compared with TGF- $\beta$ 1-only-treated cells (Fig. 2B).

The TGF- $\beta$ 1-induced EMT is mediated via the Smad- and MAPK-signaling pathways. Smad2/3, p38, and ERK are phosphorylated by

TGF- $\beta$ 1 stimulation, and the activation of these factors can induce the EMT through the regulation of the transcription factors and target genes. Snail, a transcription factor, controls the EMT through the direct repression of E-cadherin in cancer cells [3]. This study investigated whether RGE could decrease the phosphorylation of Smad2/3, p38, and ERK in the TGF- $\beta$ 1-treated HT29 cells. TGF- $\beta$ 1 treatment promoted phosphorylation of p38, ERK, as well as Smad2/3 in HT29 cells. Interestingly, the RGE treatment reduced the TGF- $\beta$ 1-induced phosphorylation of Smad2/3, p38, and ERK (Fig. 2C). Moreover, the epithelial marker E-cadherin was down-regulated and the mesenchymal marker Snail was upregulated by the TGF- $\beta$ 1 treatment. However, RGE increased the E-cadherin expression and decreased the Snail expression in the TGF- $\beta$ 1-treated HT29 cells (Fig. 2D). These results demonstrated that RGE inhibited the activation of Smad-dependent and Smad-independent MAPK-signaling pathways in the TGF- $\beta$ 1-treated HT29 cells.

### 3.3. Effects of RGE on the adhesion, migration, and invasion ability of CRC cells

To determine the effects of RGE on the metastatic abilities of CRC cells, we performed adhesion, wound-healing, and invasion assays in CT26 and HT29 cells. The adhesion, migration, and invasion abilities of CT26 cells were suppressed by RGE (50–200  $\mu\text{g}/\text{mL}$ ) treatment in a dose-dependent manner (Fig. 3). Additional



**Fig. 3.** Korean Red Ginseng extract (RGE) inhibited adhesion, migration, and invasion in CT26 cells. (A) Adhesion assay; CT26 cells ( $3 \times 10^3$  cells/well) were incubated in a Matrigel-coated 96-well plate in the presence of RGE (50–200  $\mu\text{g}/\text{mL}$ ) for 24 h. (B) Wound healing assay; CT26 cells ( $1 \times 10^6$  cells/well) were seeded in a six-well microplate and incubated until 90% confluent. The monolayers were wounded and cultured with RGE (50–200  $\mu\text{g}/\text{mL}$ ) for 24 h. (C) Invasion assay; CT26 cells ( $2.5 \times 10^4$  cells/well) were added to the upper side of a Matrigel-coated Transwell chamber with RGE (50–200  $\mu\text{g}/\text{mL}$ ) for 24 h. The inner side of the membrane was stained to confirm the invasion ability of cells.

experiments were performed to evaluate the effects of RGE on human-derived CRC cells using the TGF- $\beta$ 1-treated HT29 cells. As shown in Fig. 4, the TGF- $\beta$ 1 (10 ng/mL) treatment enhanced cancer-cell adhesion, migration, and invasion. However, RGE (200–600  $\mu$ g/mL) prevented the TGF- $\beta$ 1-induced adhesion, migration, and invasion of HT29 cells. These results indicated that RGE may inhibit metastatic abilities through the suppression of EMT in CRC cells.

3.4. Effects of RGE on the expression and activity of MMP-9 in CRC cells

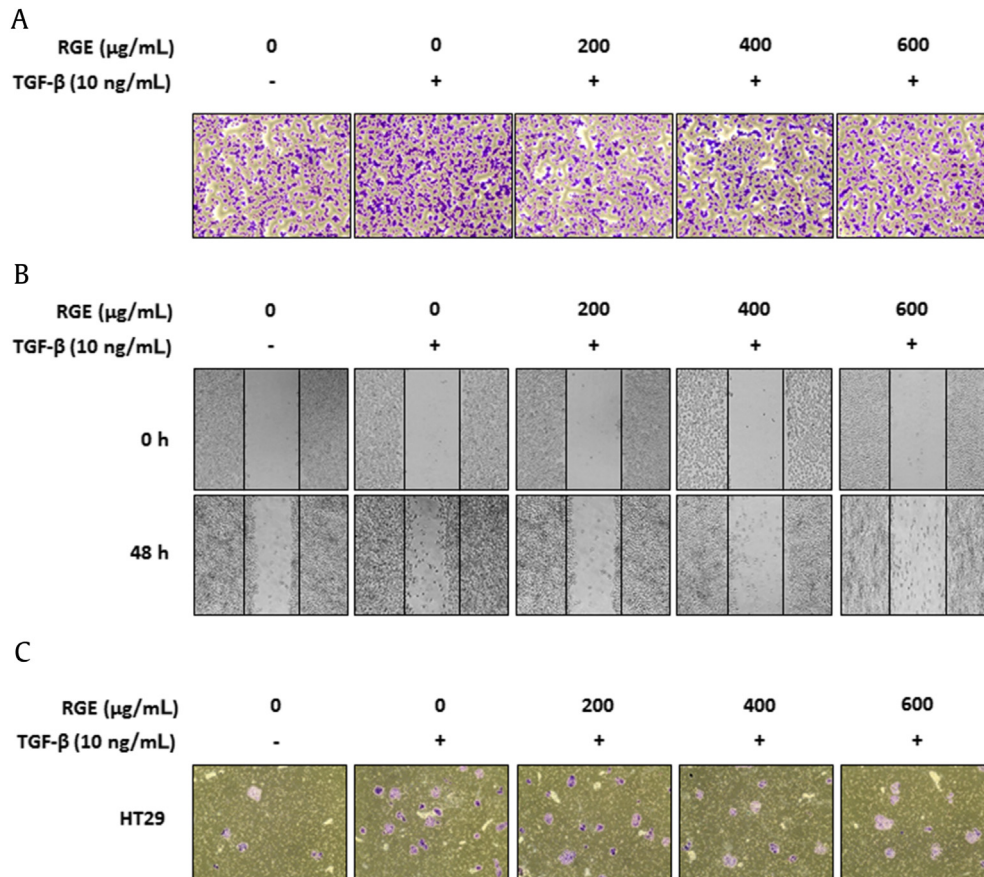
The MMP family comprises extracellular-matrix-degrading enzymes that promote the migration and invasion of cancer cells. MMP-9, a type IV collagenase, contributes to tumor progression and metastasis. TGF- $\beta$ 1 can induce the upregulation of MMP-9 in various cancer cells [20]. Therefore, we conducted zymography to detect the activity of MMP-9 after the treatment of RGE to CT26 cells. The RGE (50–200  $\mu$ g/mL) treatment suppressed the MMP-9 activity in CRC cells (Fig. 5A). Additionally, it was observed that RGE (200–600  $\mu$ g/mL) inhibited the TGF- $\beta$ 1-induced MMP-9 activity of HT29 cells (Fig. 5B). Moreover, the mRNA expression level of MMP-9 was downregulated by the RGE treatment in a dose-dependent manner in CT26 and HT29 cells (Fig. 5C and 5D). These results indicated that RGE inhibited the expression and activity of MMP-9 in CRC cells.

3.5. Effects of RGE on colorectal lung metastasis and EMT in experimental mice models

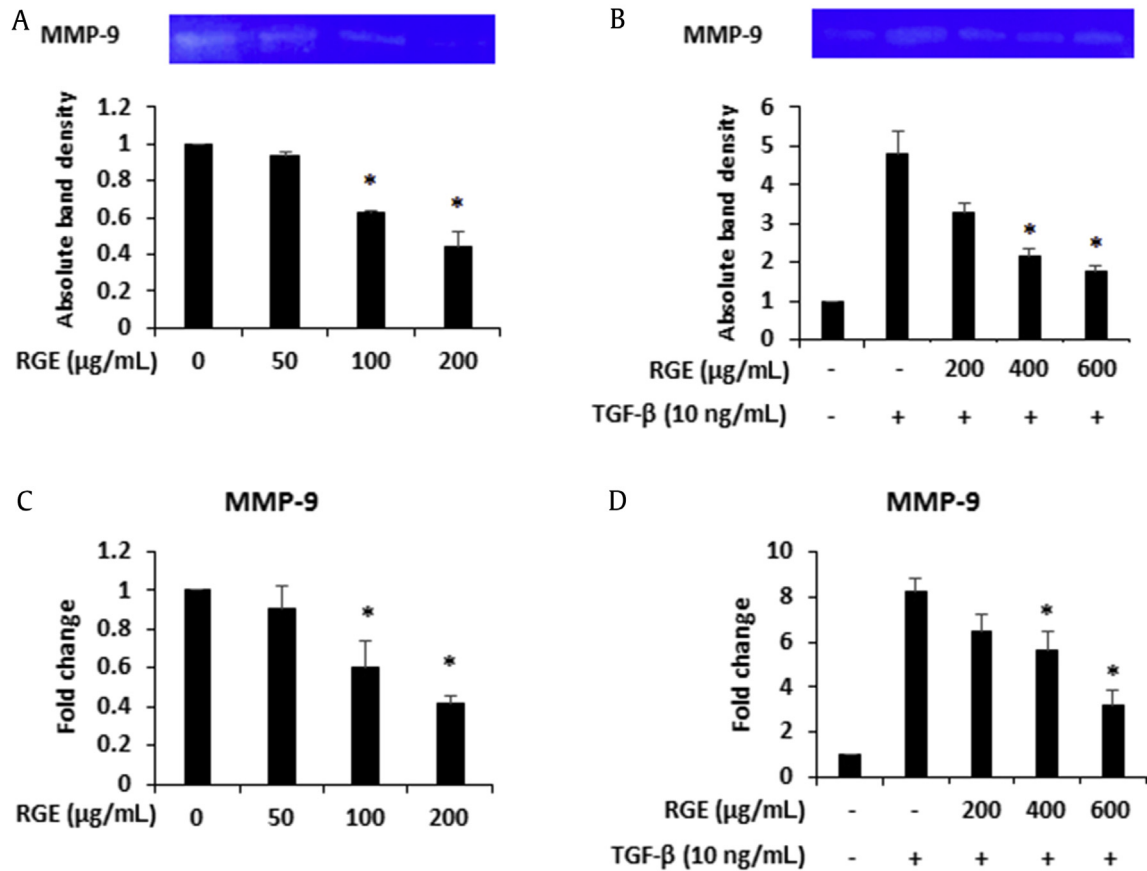
CT26 cells were intravenously injected into mice as models of lung metastasis to evaluate the antimetastatic effect of RGE in an *in vivo* experiment. As the differences between groups were not statistically significant, it was considered that the 2-wk oral administration of RGE did not exert any toxicity in mice (Table 1). RGE inhibited the lung metastasis of CT26 cells compared with the control group (Fig. 6A), and significantly decreased the number of tumor nodules (Fig. 6B). The lungs were homogenized and analyzed by Western blotting to confirm the regulatory effect of RGE on the EMT of CT26 cells. The RGE administration decreased the expression of Snail, but increased the expression of E-cadherin (Fig. 6C). Moreover, the phosphorylation of Smad2/3, p38, and ERK was decreased by the oral administration of RGE (Fig. 6E). The relative density of bands is shown in Fig. 6D and 6F. These results indicated that RGE suppressed the colorectal lung metastasis through the inhibition of the EMT in CRC cells.

4. Discussion

Approximately 60% of patients with CRC develop metastatic diseases, and the main cause of CRC-related mortality is colorectal metastasis [1]. In a previous study, it was reported that RGE reduced the proliferation, motility, and invasion of the human CRC



**Fig. 4.** Korean Red Ginseng extract (RGE) inhibited the transforming growth factor- $\beta$  (TGF- $\beta$ )-induced adhesion, migration, and invasion abilities of HT29 cells. (A) Adhesion assay; HT29 cells ( $1 \times 10^4$  cells/well) were seeded in a Matrigel-coated 96-well plate and treated with TGF- $\beta$ 1 (10 ng/mL), in the presence or absence of RGE (200–600  $\mu$ g/mL) for 24 h. (B) Wound healing assay; HT29 cells ( $2 \times 10^5$  cells/well) were plated in a six-well plate and cultured until 90% confluent. The cells were scratched with a pipette tip and incubated with TGF- $\beta$ 1 with or without RGE (200–600  $\mu$ g/mL) for 72 h. (C) Invasion assay; HT29 cells ( $5 \times 10^4$  cells/well) were seeded on the upper side of a Matrigel-coated Transwell chamber with TGF- $\beta$ 1 in the presence or absence of RGE (200–600  $\mu$ g/mL) for 72 h. The inner side of the chamber was stained with Giemsa solution.



**Fig. 5.** Korean Red Ginseng extract (RGE) reduced the expression and activity of matrix metalloproteinase (MMP)-9 in colorectal cancer cells. (A) and (B) Activity of MMP-9 from RGE-treated (A) CT26 and (B) HT29 cells. CT26 cells ( $5 \times 10^5$  cells/well) were incubated in a six-well plate with RGE (50–200 µg/mL) for 24 h. HT29 cells ( $1 \times 10^6$  cells/well) were seeded in a six-well microplate and treated with transforming growth factor-β1 (TGF-β1) in the presence or absence of RGE (200–600 µg/mL) for 72 h. The supernatant was harvested and used for gelatin zymography. (C) and (D) mRNA expression of MMP-9 in RGE-treated (C) CT26 and (D) HT29 cells. The results are expressed as the mean  $\pm$  standard deviation of three independent experiments. \* $p < 0.05$ .

cell line SW480. In addition, colorectal lung metastasis was inhibited by RGE via the decreased expression of MMP-2 and MMP-9 [16]. However, the inhibitory properties and molecular mechanisms of RGE on the EMT of CRC cells have not been investigated. This study first confirmed the RGE concentrations that did not affect the viability of CRC cells and normal cell lines. RGE (400–1,000 µg/mL) decreased the viability of CT26 cells, whereas 1,000 µg/mL RGE did not affect the viability of HT29 cells. Additionally, a maximum dose of 600 µg/mL RGE did not cause cytotoxicity in the normal cell lines CCD-18Co and RBL-2H3 cells (Fig. 1). Based on these results, the RGE treatment was administered up to concentrations of 200 µg/mL and 600 µg/mL on the CT26 and HT29 cells, respectively.

EMT is a multistage process that includes the downregulation of cell–cell adhesion and intercellular interactions, an increase in mesenchymal features, and the acquisition of migration and invasion ability. TGF-β1 is a typical inducer of EMT via the Smad-

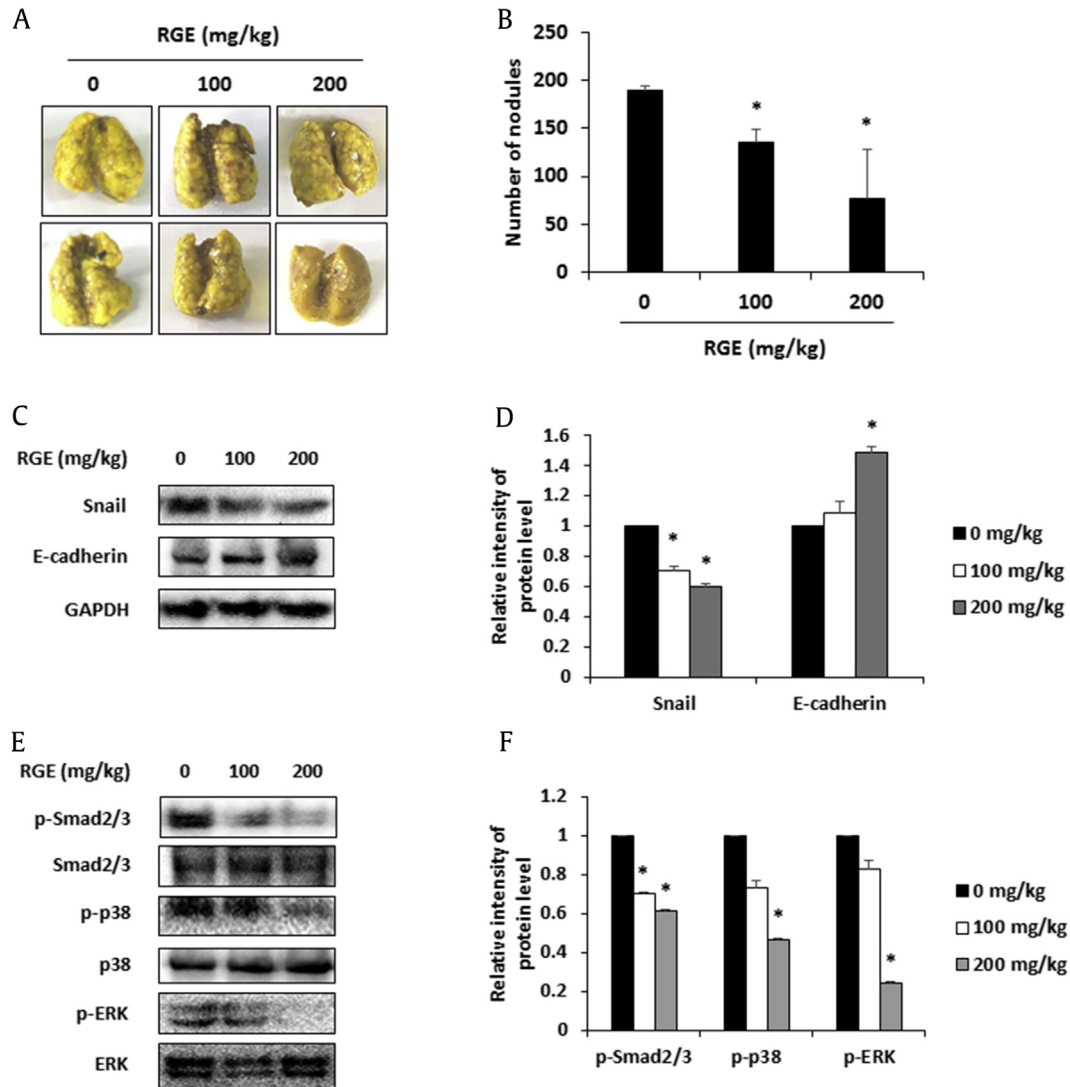
dependent or Smad-independent signaling pathway in CRC cells [21]. In this study, the TGF-β1-treated HT29 cells showed mesenchymal features, such as spindle-cell-like morphology, the downregulation of E-cadherin, and the upregulation of N-cadherin, whereas the RGE-treated cells were restored to epithelial phenotypes (Fig. 2A and 2B). Ginsenoside Rg3 (Rg3), a specific compound found in Korean Red Ginseng, exerted an inhibitory effect on the EMT in lung-cancer cells. Rg3 also inhibited the EMT of human nonsmall-cell lung carcinoma cells, including the A549, H1299, and H358 cell lines, through the downregulation of fucosyltransferase 4 [22]. Moreover, the TGF-β1-induced EMT was suppressed by Rg3 through the Smad2/p38 signaling-mediated expression of vimentin/Snail/E-cadherin in A549 cells [23]. As expected, RGE inhibited the upregulation of Snail and the downregulation of E-cadherin expression via the Smad2/3 and MAPK (ERK and p38) signaling pathways in the TGF-β1-treated HT29 cells (Fig. 2C and 2D). These inhibitory effects of RGE on the EMT via Smad-/MAPK-signaling-mediated Snail/E-cadherin expression were confirmed in the metastatic lung tissues (Fig. 6C–6F).

Metastasis is a multicellular process that involves the adhesion, migration, and invasion of cancer cells [24]. Metastatic cancer cells acquire mesenchymal phenotypes through the process of EMT, migrate directly to lymphatic and blood vessels, and release type IV collagenase MMP-2 and MMP-9 to degrade the basement membranes and trigger the invasive process [20]. Ginsenoside Rd has an antimetastatic effect on human hepatocellular carcinoma through the inhibition of the adhesion, migration, and invasion of HepG2

**Table 1**  
Effects of RGE on parameters of liver and kidney function in CT26-injected mice

Groups	ALT (IU/L)	AST (IU/L)	Creatinine (mg/dL)	BUN (mg/dL)
RGE 0 mg/kg	270.7 $\pm$ 5.6	107.2 $\pm$ 7.5	0.17 $\pm$ 0.036	22.2 $\pm$ 2.8
RGE 100 mg/kg	281.5 $\pm$ 4.4	104.8 $\pm$ 4.9	0.15 $\pm$ 0.035	20.6 $\pm$ 2.7
RGE 200 mg/kg	277 $\pm$ 6.4	106.5 $\pm$ 2.7	0.19 $\pm$ 0.039	22.8 $\pm$ 2.5

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen



**Fig. 6.** Korean Red Ginseng extract (RGE) inhibited colorectal lung metastasis through the inhibition of the epithelial–mesenchymal transition in CT26 cells. (A) and (B) The mice were allocated to three groups ( $n = 8$  each), and CT26 cells ( $1 \times 10^5$  cells) were intravenously injected at 2 h after saline RGE oral administration. RGE (100 mg/kg and 200 mg/kg) was administered once per day for 2 wk. After sacrifice, the lungs were excised and the number of nodules was counted. (A) Images of lung metastasis. (B) Number of nodules. (C) and (D) The expression of Snail and E-cadherin in lung tissue. (E) and (F) The expression of Smad2/3, and phosphorylation of p38 and ERK in lung tissue. The results are expressed as the mean  $\pm$  standard deviation of three independent experiments. \* $p < 0.05$ . ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

cells [25]. In this study, RGE inhibited the adhesion, migration, and invasion of CT26 and HT29 cells (Figs. 3 and 4). The activity and expression of MMP-9 were also decreased by the RGE treatment in both CRC cell types (Fig. 5). Moreover, the RGE administration inhibited the lung metastasis of CT26 cells through the regulation of EMT via the Smad2/3- and p38/ERK signaling pathways (Fig. 6). The RGE-induced decrease in the suppression of the EMT and the metastatic abilities of CRC cells might lead to the inhibition of colorectal metastasis.

Collectively, this study demonstrated that RGE prevented colorectal lung metastasis through the inhibition of EMT and the metastatic phenotypes of CRC cells. RGE decreased the TGF- $\beta$ 1-induced EMT, such as mesenchymal-like morphologic changes by regulation of the expression of E-cadherin and Snail via the Smad/p38/ERK-signaling pathway. Additionally, the metastatic abilities of CRC cells, including adhesion, migration, and invasion, were reduced by the RGE treatment. These results indicated the potential of RGE as a novel chemopreventive agent and adjuvant medicine for colorectal metastasis.

## Conflicts of interest

The authors have no conflicts of interest to declare.

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## References

- [1] DeSantis CE, Lin CC, Mariotto AB, Siegel RL, Stein KD, Kramer JL, Alteri R, Robbins AS, Jemal A. Cancer treatment and survivorship statistics. *CA Cancer Clin* 2014;64:252–71.
- [2] Jung KW, Won YJ, Kong HJ, Oh CM, Lee DH, Lee JS. Cancer statistics in Korea: incidence, mortality, survival, and prevalence in 2011. *Cancer Res Treat* 2014;46:109–23.
- [3] Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial–mesenchymal transition. *Nat Rev Mol Cell Biol* 2014;15:178–96.
- [4] Huang A, Gilmour JW, Imami N, Amjadi P, Henderson DC, Allen-Mersh TG. Increased serum transforming growth factor-beta1 in human colorectal



- cancer correlates with reduced circulating dendritic cells and increased colonic Langerhans cell infiltration. *Clin Exp Immunol* 2003;134:270–8.
- [5] Calon A, Espinet E, Palomo-Ponce S, Tauriello DV, Iglesias M, Céspedes MV, Sevillano M, Nadal C, Jung P, Zhang XH, et al. Dependency of colorectal cancer on a TGF- $\beta$ -driven program in stromal cells for metastasis initiation. *Cancer Cell* 2012;22:571–84.
- [6] Heldin CH, Vanlandewijck M, Moustakas A. Regulation of EMT by TGF $\beta$  in cancer. *FEBS Lett* 2012;586:1959–70.
- [7] Jeon C, Kang S, Park S, Lim K, Hwang KW, Min H. Cell stimulatory effects of Korean Red Ginseng through modulation of myeloid-derived suppressor cells. *J Ginseng Res* 2011;35:462–70.
- [8] Choi JY, Woo TS, Yoon SY, Campomayor dela Peña I, Choi YJ, Ahn HS, Lee YS, Yu GY, Cheong JH. Red ginseng supplementation more effectively alleviates psychological than physical fatigue. *J Ginseng Res* 2011;35:331–8.
- [9] Kee JY, Jeon YD, Kim DS, Han YH, Park J, Youn DH, Kim SJ, Ahn KS, Um JY, Hong SH. Korean Red Ginseng improves atopic dermatitis-like skin lesions by suppressing expression of proinflammatory cytokines and chemokines *in vivo* and *in vitro*. *J Ginseng Res* 2017;41:134–43.
- [10] Park JG, Son YJ, Aravinthan A, Kim JH, Cho JY. Korean Red Ginseng water extract arrests growth of xenografted lymphoma cells. *J Ginseng Res* 2016;40:431–6.
- [11] Lee SM, Bae BS, Park HW, Ahn NG, Cho BG, Cho YL, Kwak YS. Characterization of Korean Red Ginseng (*Panax ginseng* Meyer): history, preparation method, and chemical composition. *J Ginseng Res* 2015;39:384–91.
- [12] Chen XJ, Liu WJ, Wen ML, Liang H, Wu SM, Zhu YZ, Zhao JY, Dong XQ, Li MG, Bian L, et al. Ameliorative effects of compound K and ginsenoside Rh1 on non-alcoholic fatty liver disease in rats. *Sci Rep* 2017;7. <http://dx.doi.org/10.1038/srep41144>.
- [13] Li J, Du J, Liu D, Cheng B, Fang F, Weng L, Wang C, Ling C. Ginsenoside Rh1 potentiates dexamethasone's anti-inflammatory effects for chronic inflammatory disease by reversing dexamethasone-induced resistance. *Arthritis Res Ther* 2014;16. <http://dx.doi.org/10.1186/ar4556>.
- [14] Yang J, Yuan D, Xing T, Su H, Zhang S, Wen J, Bai Q, Dang D. Ginsenoside Rh2 inhibiting HCT116 colon cancer cell proliferation through blocking PDZ-binding kinase/T-LAK cell-originated protein kinase. *J Ginseng Res* 2016;40:400–8.
- [15] Kim MJ, Yun H, Kim DH, Kang I, Choe W, Kim SS, Ha J. AMP-activated protein kinase determines apoptotic sensitivity of cancer cells to ginsenoside-Rh2. *J Ginseng Res* 2014;38:16–21.
- [16] Seo EY, Kim WK. Red ginseng extract reduced metastasis of colon cancer cells *in vitro* and *in vivo*. *J Ginseng Res* 2011;35:315–24.
- [17] Ho YL, Li KC, Chao W, Chang YS, Huang GJ. Korean Red Ginseng suppresses metastasis of human hepatoma SK-Hep1 cells by inhibiting matrix metalloproteinase-2/-9 and urokinase plasminogen activator. *Evid Based Complement Alternat Med* 2012;2012. <http://dx.doi.org/10.1155/2012/965846>.
- [18] Mochizuki M, Yoo YC, Matsuzawa K, Sato K, Saiki I, Tono-oka S, Samukawa K, Azuma I. Inhibitory effect of tumor metastasis in mice by saponins, ginsenoside-Rb2, 20(R)- and 20(S)-ginsenoside-Rg3, of red ginseng. *Biol Pharm Bull* 1995;18:1197–202.
- [19] Shibata S. Chemistry and cancer preventing activities of ginseng saponins and some related triterpenoid compounds. *J Korean Med Sci* 2001;16(Suppl):S28–37.
- [20] Gialeli C, Theocharis AD, Karamanos NK. Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting. *FEBS J* 2011;278:16–27.
- [21] Bates RC, Mercurio AM. The epithelial–mesenchymal transition (EMT) and colorectal cancer progression. *Cancer Biol Ther* 2005;4:365–70.
- [22] Tian L, Shen D, Li X, Shan X, Wang X, Yan Q, Liu J. Ginsenoside Rg3 inhibits epithelial–mesenchymal transition (EMT) and invasion of lung cancer by down-regulating FUT4. *Oncotarget* 2016;7:1619–32.
- [23] Kim YJ, Choi WI, Jeon BN, Choi KC, Kim K, Kim TJ, Ham J, Jang HJ, Kang KS, Ko H. Stereospecific effects of ginsenoside 20-Rg3 inhibits TGF- $\beta$ 1-induced epithelial–mesenchymal transition and suppresses lung cancer migration, invasion and anoikis resistance. *Toxicology* 2014;322:23–33.
- [24] Yilmaz M, Christofori G, Lehenbre F. Distinct mechanisms of tumor invasion and metastasis. *Trends Mol Med* 2007;13:535–41.
- [25] Yoon JH, Choi YJ, Cha SW, Lee SG. Anti-metastatic effects of ginsenoside Rd via inactivation of MAPK signaling and induction of focal adhesion formation. *Phytomedicine* 2012;19:284–92.