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4-Shogaol, an Active Constituent of Dietary Ginger, Inhibits Metastasis of MDA-MB-231 Human Breast Adenocarcinoma Cells by Decreasing the Repression of NF-κB/Snail on RKIP

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ABSTRACT: 4-Shogaol is one of the phytoconstituents isolated from dried red ginger, which is commercially available to consumers. Some active constituents from ginger have been found to have anti-inflammatory and antioxidant effects, but studies on 4-shogaol have been relatively rare. This is the first report describing the antimetastasis activities of 4-shogaol and the possible mechanisms. This study determined that 4-shogaol inhibits the migration and invasion of MDA-MB-231 and causes mesenchymal−epithelial transition (MET). In addition, 4-shogaol suppresses the activation of NF-κB and cell migration and invasion induced by TNF-α. Furthermore, 4-shogaol has been shown to inhibit the phosphorylation of IκB and the translocation of NF-κB/Snail in MDA-MB-231. This study shows that RKIP, an inhibitory molecule of IKK, is up-regulated after 4-shogaol treatment and prolongs the inhibitory effects of 4-shogaol. Inhibition of RKIP by shRNA transfection significantly decreases the inhibitory effect of 4-shogaol on the NF-κB/Snail pathway, together with cell migration and invasion, whereas overexpression of Snail suppresses 4-shogaol-mediated metastasis inhibition and E-cadherin upregulation. Finally, the animal model revealed that 4-shogaol effectively inhibits metastasis of MDA-MB-231 in mice. This study demonstrates that 4-shogaol may be a novel anticancer agent for the the treatment of metastasis in breast cancer.

KEYWORDS: *4-shogaol, red ginger, metastasis, breast cancer*

INTRODUCTION

Breast cancer, the most common female cancer, comprises 23% of all cancers, with >1 million new cases each year.[1] About 30−40% of women with breast cancer will develop metastasis, which is defined as breast tumor cells' migration to, and invasion of, other organs such as the lungs, liver, and bones.[2] Metastasized breast cancer is particularly challenging because it is highly resistant to radiation and conventional chemotherapeutic agents.[2] Consequently, novel therapeutic agents are needed to deal with the disease's increasing incidence, to advance the efficacy of chemotherapy in human breast cancer, and to increase the number of alternative regimens.

Epithelial−mesenchymal transition (EMT) is a critical process in cancer development, enhancing cancer cells' ability to metastasize.[3] Snail, one of the important transcription factors involved in the regulation of EMT, acts by repressing the expression of E-cadherin and claudin-3 genes.[4] Snail expression and function is regulated by several signaling pathways, such as p21-activated kinase, integrin-linked kinase signaling 1, and nuclear factor κB (NF-κB). Activated NF-κB signaling, induced by various growth factors, inflammatory factors, or genetic transfection, promotes cancer invasion and metastasis by enhancing Snail stabilization or expression.[5−7] NF-κB has been implicated in the overexpression or overactivation of breast cancer cells and is involved in every stage of cancer development.[8,9] Furthermore, inhibition of NF-κB by genetic knockdown or chemical reagents decreased cancer cell proliferation, invasion, and metastasis and ameliorated the chemoresistance of cancer to anticancer therapies.[10−12] Inhibition of NF-κB suppresses tumor cell metastasis through inhibition of the downstream target Snail and by induction of Raf kinase inhibitor protein (RKIP).[13] In addition, RKIP directly reduces IκB kinase (IKK) activity, thus forming a negative feedback loop on the activation of NF-κB and Snail.[13−16]

Ginger (*Zingiber officinale*) is a well-known plant used in cooking worldwide and has long been reputed to have medicinal properties. It is a herbaceous, rhizomatous perennial plant

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widely distributed throughout tropical and subtropical regions[17] and is cultivated on a large scale in Nigeria, India, Bangladesh, Sri Lanka, Taiwan, and other East Asian countries.[17] Ginger has been used since antiquity for a wide array of unrelated ailments, such as arthritis, rheumatism, sprains, muscular aches, pains, sore throats, cramps, constipation, indigestion, vomiting, hypertension, dementia, fever, infectious diseases, and helminthiasis.[18−20] 6-Shogaol, an active ingredient in ginger, has also been shown to have anti-inflammatory and anticancer effects,[21−26] but this study is the first to determine the anticancer activity of 4-shogaol, one of the novel components of dietary ginger, on cell migration and invasion in human breast cancer, both in vitro and in vivo. We have found that 4-shogaol effectively inhibits the metastasis of breast cancer by decreasing NF-κB and Snail, sequentially resulting in the reinforcement of RKIP expression and inhibition of cell migration and invasion.

MATERIALS AND METHODS

Test Compound. The rhizomes (27.5 kg) of *Z. officinale* were chipped, air-dried, and extracted repeatedly with MeOH (50 L × 4) at room temperature. The combined MeOH extracts (905.5 g) were then evaporated and further separated into six fractions by column chromatography (CC) on silica gel (4.7 kg, 70−230 mesh) with a gradient of *n*-hexane/CH_2Cl_2/acetone. Fraction 2 (83.3 g) was eluted with *n*-hexane/acetone (60:1). The fraction was next subjected to silica gel CC (*n*-hexane/acetone mixtures) and yielded 4-shogaol (241 mg). The purity of 4-shogaol exceeded 95%, as determined by HPLC, and its chemical structure was confirmed by NMR.[27]

Cell Culture and Cell Viability Assay. MDA-MB-231 human breast adenocarcinoma cells (ATCC HTB-26) were cultured in MEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FBS, 0.1 mg/mL streptomycin, and 100 units/mL penicillin (Life Technologies, Inc.). Cell viability was assessed by Premixed WST-1 Cell Proliferation Reagent (Clontech Laboratories Inc., Mountain View, CA) according to the manufacturer's instructions.

Cell Migration and Invasion Assay. A cell migration and invasion assay was conducted using a QCM 24-well Cell Migration Assay and Invasion System. Briefly, 3×10^4 cells were seeded into the top chamber and treated with different concentrations of 4-shogaol. Ten percent FBS or TNF-α was added to the bottom wells for 24 h as chemoattractant. At the end of the treatment, cells were poststained with CyQuant GR dye in cell lysis buffer for 15 min at room temperature. Then, fluorescence of the invaded cells was read using a fluorescence plate reader at excitation/emission wavelengths of 485/520 nm.

Scratch Wound-Healing Assay. MDA-MB-231 cells were allowed to grow into full confluence in 24-well plates. The following day, a uniform scratch was made down the center of the well using a micropipet tip, followed by washing once with PBS. Vehicle control and various concentrations of 4-shogaol were added to the respective wells for the indicated times. Photographic imaging was performed using the Nikon inverted microscope.

Immunoblot/Immunoprecipitation. Cells (8×10^6/dish) were seeded in a 10 cm dish. After 24 h of incubation, the cells were treated with various concentrations of 4-shogaol for the indicated times. Total cell extracts were prepared in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF, 5 μg/mL aprotinin, 5 μg/mL leupetin). Equivalent amounts of protein were resolved by SDS-PAGE and transferred to PVDF membranes. After the membranes were blocked in Tris-buffer saline containing 0.05% Tween 20 (TBST) and 5% nonfat powdered milk, they were incubated with primary antibodies at 4 °C for 1−16 h. After three washings with TBST for 10 min each, the membranes were incubated with horseradish peroxidase-labeled secondary antibody for 1 h and then washed again. Detection was performed using an enhanced chemiluminescence blotting detection system (Amersham, USA).

For IP, cell lysates (200 μg of total protein) were incubated with 2 μg of anti-IKKα overnight and then with 20 μL of protein A-agarose beads (Millipore, Bedford, MA) for 2 h at 4 °C. Association of IKKα with RKIP was detected by incubating the blots with anti-RKIP antibodies (Cell Signaling).

NF-κB DNA Binding Assay. NF-κB activity was determined by a Trans-AM ELISA kit, which was used according to the manufacturer's specifications (Active Motif, Carlsbad, CA). Briefly, the transcriptional factor of nuclear extracts, which were prepared by using a Nuclear Extract kit (Active Motif), were captured by binding to a consensus oligonucleotide (5′-GGGACTTTCC-3′) immobilized on a 96-well plate. The p65 subunit of NF-κB was determined in a colorimetric reaction using specific primary antibody and a secondary horseradish peroxidase-conjugated antibody. Spectrophotometric data were expressed as the ratio of absorbance of each experimental condition compared with control cells exposed to vehicle alone.

Real-Time PCR. RNA isolation was performed using the TRIzol reagent (Invitrogen). cDNA was prepared using an oligo(dT) primer and reverse transcriptase (Takara, Shiga, Japan) following standard protocols. Real-time PCR was performed using SYBR Green on the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Each PCR reaction mixture contained 200 nM of each primer, 10 μL of 2× SYBR Green PCR Master Mix (Applied Biosystems), 5 μL of cDNA, and RNase-free water with a total volume of 20 μL. The PCR reaction was carried out with a denaturation step at 95 °C for 10 min and then for 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All PCRs were performed in triplicate and normalized to internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Relative expression was presented using the $2^{-\triangle\triangle CT}$ method.

Gene Knockdown and Overexpression. Knockdown of RKIP in MDA-MB-231 was performed using a lentiviral expression system provided by the National RNAi Core Facility (Taipei, Taiwan). Lentiviruses were produced by cotransfecting HEK293T with pLKO-AS2 or pLKO-AS2-RKIP shRNA and two packaging plasmids (pCMVVDR8.91 and pMD.G). The changes of RKIP were measured by real-time PCR as described above. RKIP-knockdown MDA-MB-231 stable colonies were established by puromycin selection. MDA-MB-231 cells were nucleofected (Amaxa) with 0.5 μg of either the SNAIL-expressing plasmid or control plasmid. At 24 h post-transfection, Snail cDNA overexpressing-MDA-MB-231 stable colonies were established by G418 selection.

Lung Metastasis of Breast Cancer in Vivo. We implanted MDA-MB-231 (2×10^6) into mice by tail vein injection and then randomly divided the mice into two groups. The mice in the 4-shogal-treated group were intraperitoneally (ip) injected daily with 4-shogaol in a clear solution containing 4% Cremophor EL (40 mg/kg of body weight), at a volume of 0.2 mL. The control group was treated with an equal volume of vehicle. Tumor-bearing mice were sacrificed 35 days after transplantation.

Immunofluorescence and HE Staining. Noncancerous and cancerous lung tissues obtained from mice were embedded in OCT and frozen in liquid nitrogen. Sections (3−5 μm) were fixed with acetone at −20 °C and then stained using anti-NF-κB and Snail antibody (1:100, Abcam, U.K.). After washing with PBS containing 0.1% Tween-20 (PBST), slides were incubated with Dylight 549-conjugated secondary antibodies (Rockland, Gilbertsville, PA), with DAPI, for 1 h at room temperature. Data were analyzed with a confocal laser scanning microscope (Fluoview FV500, Olympus, Tokyo, Japan). Lungs were also fixed in 10% buffered formaldehyde and then embedded in paraffin. Sections were mounted on glass slides and stained with hematoxylin and eosin (H&E).

Statistical Analyses. Data were expressed as the mean ± SD of three determinations. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences ($p < 0.05$) between the means of the two test groups were analyzed by Student's *t* test post hoc.

RESULTS

4-Shogaol Decreases Cell Migration and Invasion and Increases MET in MDA-MB-231 Cells. We first assessed the effects of ginger-derived compounds on the viability of MDA-MB-231 cells. As shown in Figure 1A, all compounds (4-shogaol,

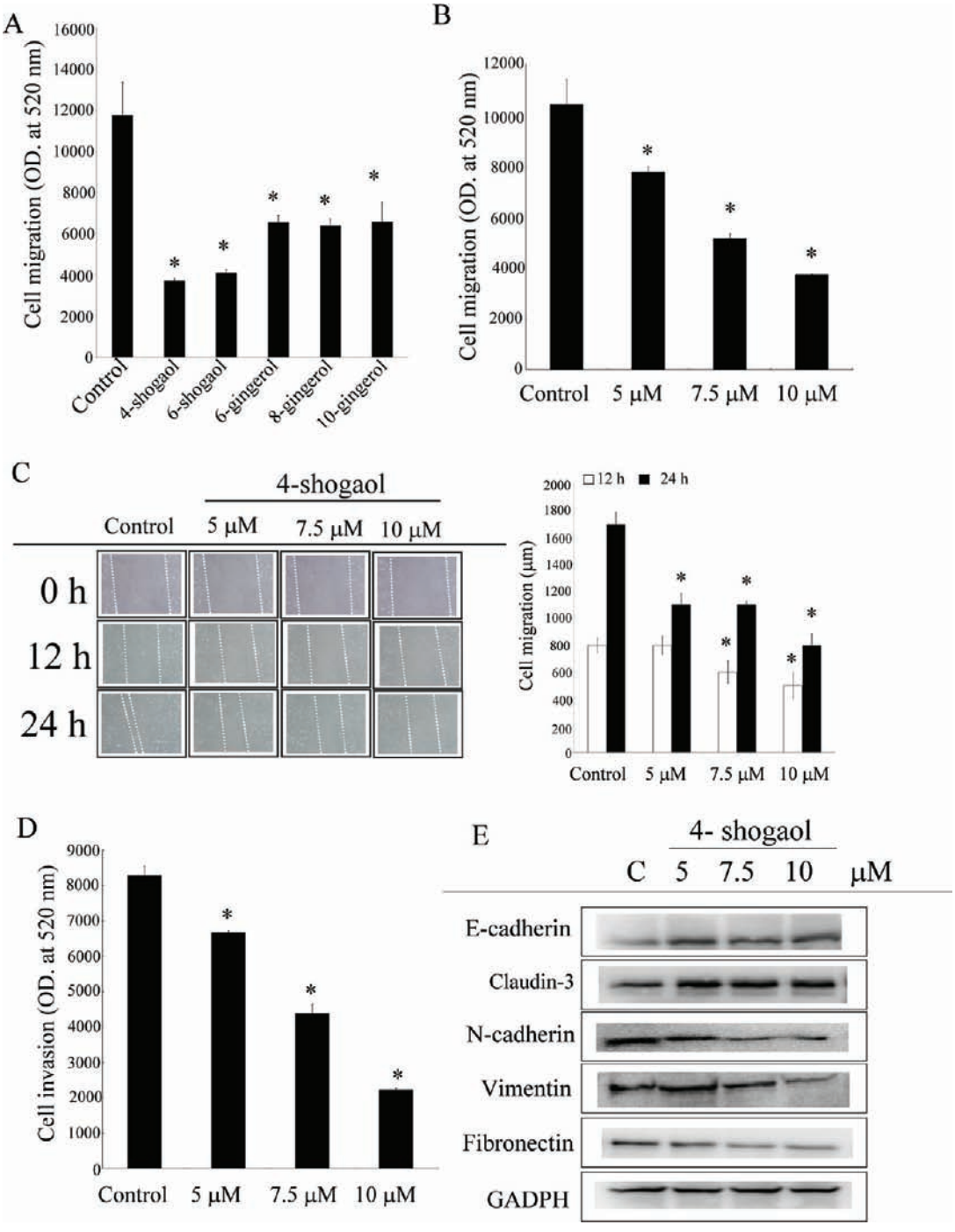

Figure 1. 4-Shogaol decreases cell migration and invasion and increases MET in MDA-MB-231 cells: (A) ginger-derived compounds (10 μM) decrease migration of MDA-MB-231 cells; (B) 4-shogaol inhibits cell migration in a dose-dependent manner, as determined by the transwell system; (C) 4-shogaol inhibits cell migration, as determined by wound healing assay; (D) 4-shogaol inhibits cell invasion in a dose-dependent manner, as determined by the transwell system; (E) 4-shogaol promotes MET in MDA-MB-231 cells. The migration and invasion abilities of MDA-MB-231 cells were quantified by QCM 24-well Cell Migration and Invasion assay kits, as described under Materials and Methods. Ten percent FBS acts as a chemoattractant of cancer migration and invasion. Cells were treated with 4-shogaol for 24 h, and the protein expression was assessed by immunoblot assay. All results are representative of at least three independent experiments. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between the two test groups, as analyzed by Student's *t* test post hoc ($p < 0.05$).

6-shogaol, 6-gingerol, 8-gingerol, and 10-gingerol) exhibit inhibitory effects on the migration of MDA-MB-231 cells. However, the inhibitory effects of 4-shogaol and 6-shogaol are greater than those of 6-gingerol, 8-gingerol, and 10-gingerol. This finding is similar to other studies with regard to the anticancer, antioxidant, and anti-inflammatory effects of gingerol.[19,26]

The α,β-unsaturated carbonyl group in shogaol may influence the conformation of the molecule, thereby modulating its inhibitory effect. This hypothesis could provide new information for the design of anticancer agents and future study of these functional groups.[19,26] We also assessed the effect of 4-shogaol on the viability of MDA-MB-231 cells. 4-Shogaol did not affect

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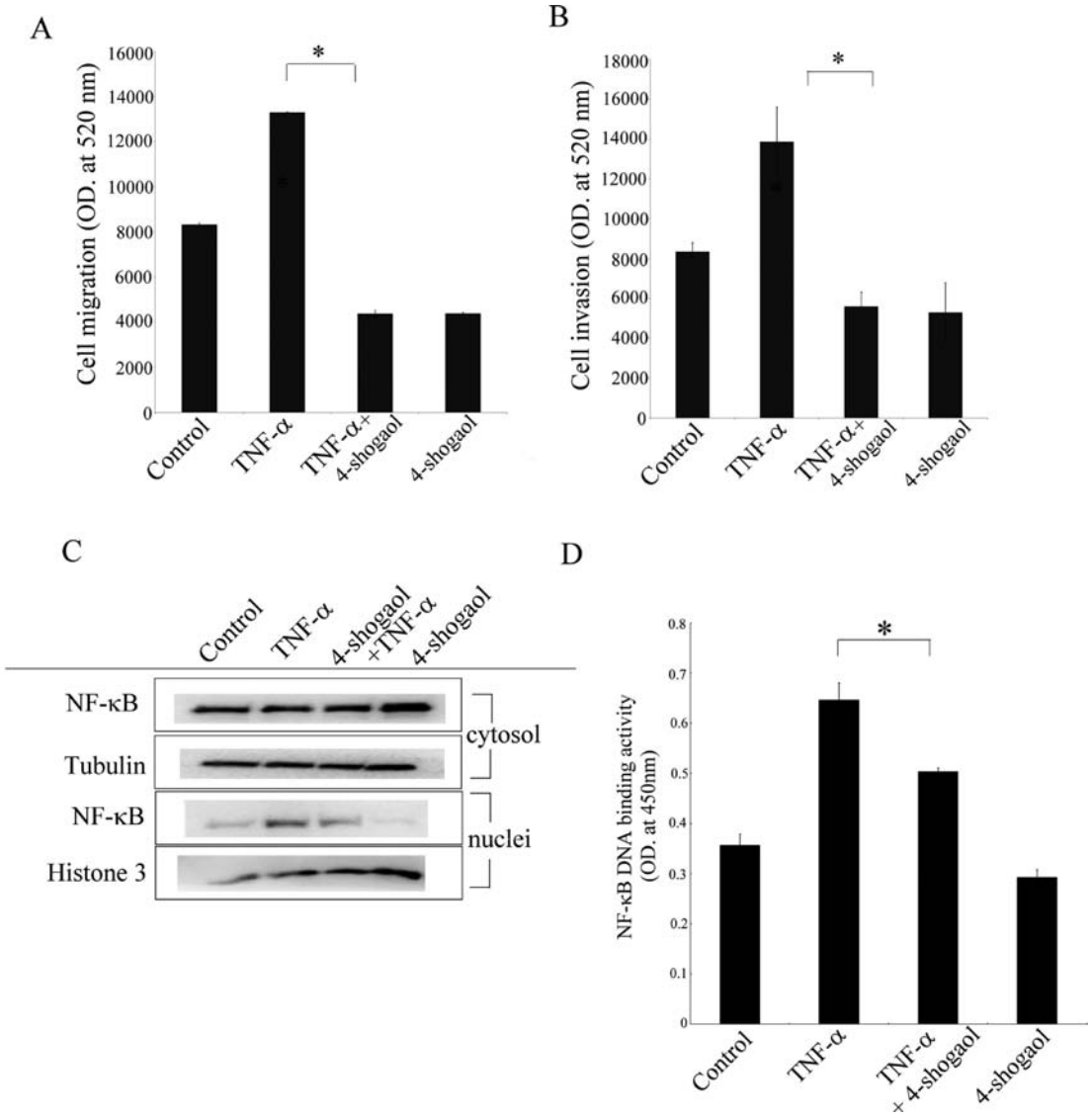

Figure 2. 4-Shogaol inhibits inducible NF-κB translocation and DNA-binding activity in MDA-MB-231 cells: 4-shogaol inhibits TNF-α-mediated cell migration (A) and invasion (B); 4-shogaol reduces TNF-α-induced nuclear translocation (C) and DNA-binding (D) of NF-κB. Cells were pretreated with 4-shogaol (7.5 μM) for 1 h, and then TNF-α (20 ng/mL) was added for another 6 h. Nuclear and cytoplasm fractions were separated by nuclear extract kit, and protein expression was assessed by immunoblot assay. The DNA binding activity of NF-κB in the nuclear fraction was assessed by Trans-AM ELISA kit. The migration and invasive abilities of MDA-MB-231 cells were quantified by QCM 24-well Cell Migration and Invasion assay kits. TNF-α (20 ng/mL) acts as a chemoattractant of cancer migration and invasion. All results are representative of at least three independent experiments. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between the two test groups, as analyzed by Student's *t* test post hoc ($p < 0.05$).

the viability of MDA-MB-231 cells at concentrations ranging from 5 to 10 μM (data not shown).

To examine the effect of 4-shogaol on human breast cancer cell migration, we employed transwell migration and wound-healing assay to characterize the cells' migration response to 4-shogaol. As shown in Figure 1B, culture medium increased the migration of MDA-MB-231 cells after 24 h of incubation, whereas 4-shogaol treatment decreased the migration of MDA-MB-231 cells in a dose-dependent manner. Furthermore, a scratch wound-healing assay also showed that 4-shogaol decreased MDA-MB-231 cells' migration ability after treatment for 12 and 24 h (Figure 1C).

Next, we also assessed the effect of 4-shogaol on breast cancer cell invasion. Compared to vehicle-treated cells, culture medium increased MDA-MB-231 cells' invasion capability. However, 4-shogaol treatment attenuated cell invasion in a dose-dependent manner after treatment for 48 h (Figure 1D).

Epithelial−mesenchymal transition (EMT) plays a crucial role in progression in the development of invasive cancer cells.[4] We assessed the effect of 4-shogaol on EMT markers in MDA-MB-231. 4-Shogaol treatment increased epithelial marker E-cadherin and claudin-3 levels and decreased the mesenchymal markers fibronectin, vimentin, and N-cadherin (Figure 1E).

4-Shogaol Inhibits Inflammatory TNF-α Induced Activation of NF-κB in MDA-MB-231 Cells. We investigated whether 4-shogaol also inhibited inflammatory cytokine TNF-α-mediated NF-κB activation and Snail expression. As shown in Figure 2A,B, TNF-α increased cell migration and invasion in MDA-MB-231 cells, whereas 4-shogaol decreased this enhancement of TNF-α on cell migration and invasion.

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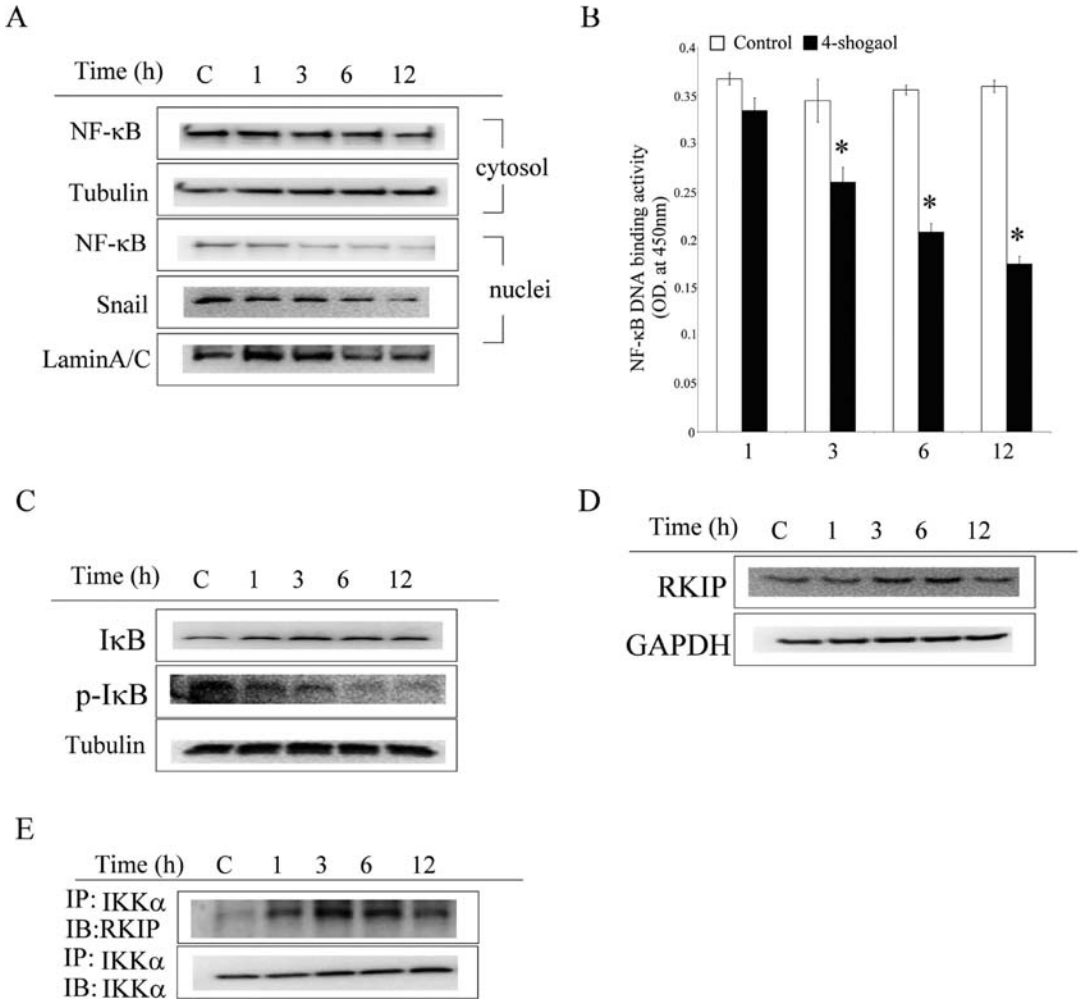

Figure 3. 4-Shogaol inhibits NF-κB/Snail signaling and increases RKIP expression in MDA-MB-231 cells: (A) 4-shogaol attenuates the amount of NF-κB and Snail in nuclei; 4-shogaol decreases NF-κB DNA binding activity (B) and IκB phosphorylation (C); 4-shogaol increases RKIP expression (D) and the interaction of RKIP with IKKα (E). Cells were treated with 4-shogaol (7.5 μM) for the indicated times, and then nuclear and cytoplasm fractions were separated by nuclear extract kit and protein expression was assessed by immunoblot assay. The DNA binding activity of NF-κB in the nuclear fraction was assessed by Trans-AM ELISA kit. The interaction of IKKα with RKIP was assessed by immunoprecipitation. All results are representative of at least three independent experiments. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between the two test groups, as analyzed by Student's *t* test post hoc ($p < 0.05$).

We further assessed whether 4-shogaol decreased TNF-α-mediated NF-κB translocation and activities. As shown in Figure 2C, treatment of MDA-MB-231 cells with TNF-α (20 ng/mL) increased the translocation of NF-κB, but this effect was abrogated by 4-shogaol. In addition, the enhancement of NF-κB DNA binding activity by TNF-α was also inhibited by 4-shogaol (Figure 2D).

4-Shogaol Inhibits NF-κB/Snail Signaling and Increases RKIP Expression in MDA-MB-231 Cells. We investigated whether NF-κB and Snail, important transcription factors in regulating cell migration and EMT, were involved in 4-shogaol-mediated inhibition of metastasis. Immunoblot analysis revealed that the protein amounts of NF-κB and Snail in the nuclei were decreased by 4-shogaol treatment (Figure 3A). In addition, NF-κB activity (nuclear fraction) decreased in a time-dependent manner in MDA-MB-231 cells after 4-shogaol treatment (Figure 3B). Because phosphorylation and degradation of IκB by IKK plays a critical role in the process of NF-κB activation,[8] we assessed the effect of 4-shogaol on the status of IκB. As shown in Figure 3C, 4-shogaol decreased the phosphorylation of IκB and increased the amount of IκB in MDA-MB-231 cells, suggesting that 4-shogaol affects NF-κB by decreasing IκB phosphorylation.

RKIP that is regulated by NF-κB and Snail has been reported, and it inhibits IKK activity by interacting with IKK to form an inactive complex.[14] Therefore, we sought to determine whether the up-regulation of RKIP is involved in the 4-shogaol-mediated decrease of NF-κB activity. As shown in Figure 3D, 4-shogaol increased the expression of RKIP protein in MDA-MB-231 cells and also distinctly increased the interaction of RKIP with IKKα after 6 h (Figure 3E).

Role of RKIP in 4-Shogaol-Mediated Inhibition of NF-κB. To analyze the role of RKIP in 4-shogaol-mediated inhibition of cancer migration and invasion, we examined the inhibition of RKIP by shRNA on cell migration and invasion. As shown in Figure 4A, RKIP shRNA inhibits the expression of RKIP at mRNA and protein levels in MDA-MB-231 cells. After 6 h of treatment, RKIP shRNA significantly reduced the inhibitory effect of 4-shogaol on the nuclear translocation of NF-κB and Snail, as well as the phosphorylation of IκB. However, RKIP shRNA did not influence the effects of 4-shogaol treatment within 3 h (Figure 4B). In addition, knockdown of RKIP

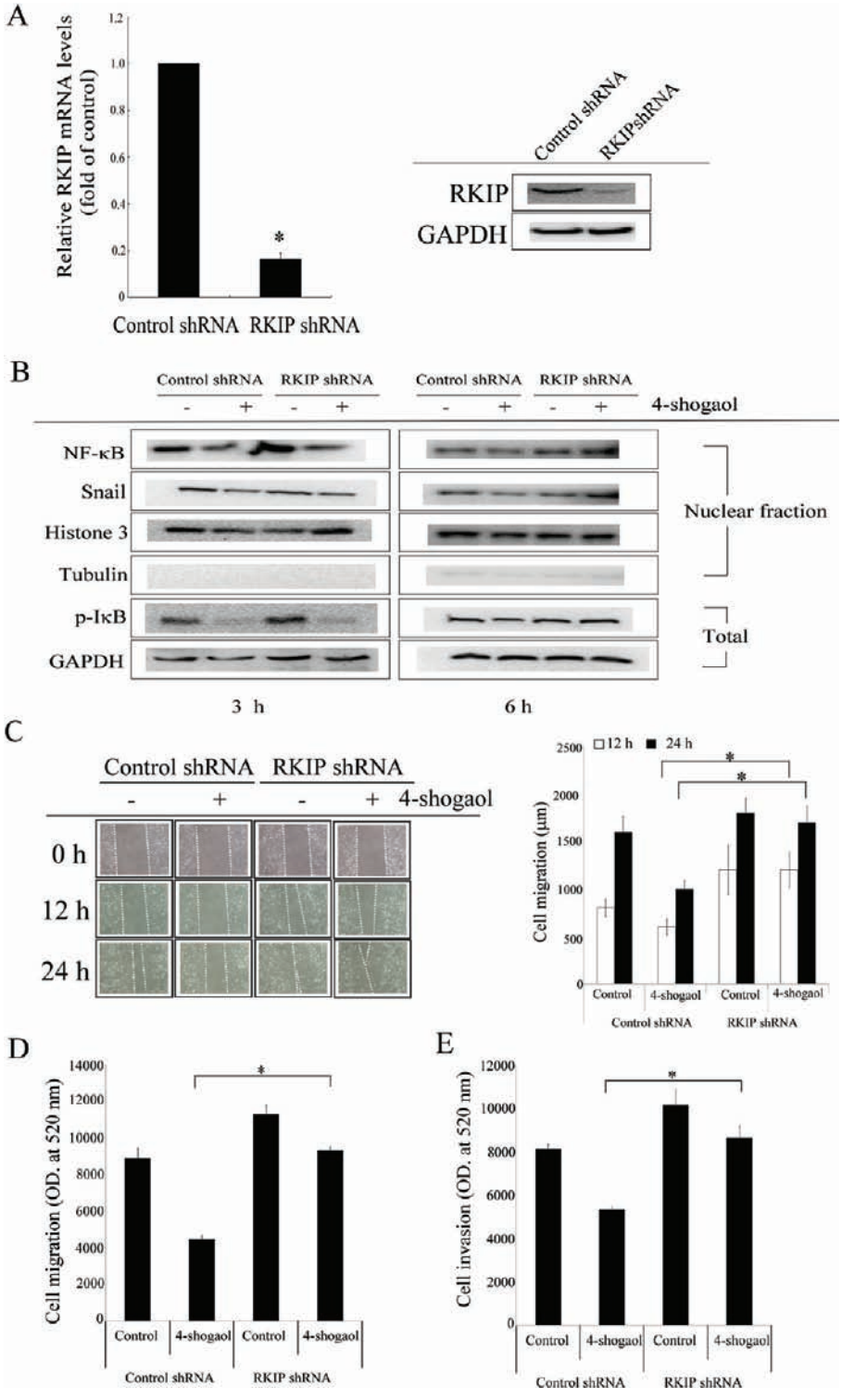

Figure 4. Role of RKIP in 4-shogaol-mediated inhibition of NF-κB: (A) RKIP shRNA decreases the amount of RKIP at mRNA and protein levels; (B) inhibition of RKIP decreases the inhibitory effect of 4-shogaol on NF-κB and Snail levels in cell nuclei, as well as IκB phosphorylation; (C) RKIP shRNA inhibits the effect of 4-shogaol on cell migration; the transwell assay also found that RKIP shRNA decreases 4-shogaol-mediated cell migration (D) and invasion (E) inhibition. Cells were treated with 4-shogaol (7.5 μM) for the indicated times, and various protein levels were assessed by immunoblot assay. The migration ability of MDA-MB-231 cells was determined by wound-healing assay and the transwell system. All results are representative of at least three independent experiments, and each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between the two test groups, as analyzed by Student's *t* test post hoc ($p < 0.05$).

also decreased the inhibitory effect of 4-shogaol on cell migration and invasion (Figure 4C–E).

Role of Snail in 4-Shogaol-Mediated Inhibition of Cell Migration and MET. To investigate the role of Snail in 4-shogaol-mediated RKIP increase and inhibition of cancer migration and promotion of MET, we examined the effect of 4-shogaol on Snail-overexpressing MDA-MB-231 cells (Figure 5A). As shown in Figure 5B–D, Snail overexpression blocks the effects

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of 4-shogaol on MDA-MB-231 cell migration and invasion. In addition, the effects of 4-shogaol in up-regulation of RKIP and E-cadherin are also abrogated at both mRNA and protein levels by Snail overexpression (Figure 5E,F).

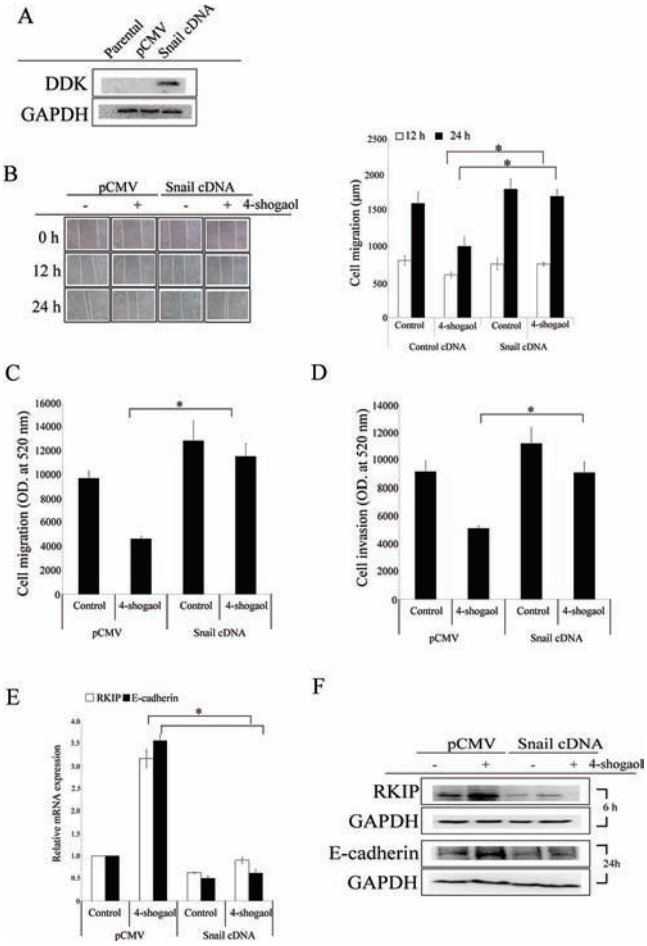

Figure 5. Role of Snail on 4-shogaol-mediated inhibition of cell migration and MET: (A) expression of exogenous Snail in pSnail plasmid-transfected MDA-MB-231 cells; overexpression of Snail decreases the inhibitory effect of 4-shogaol on cell migration (B); the transwell assay also found that Snail overexpression decreases 4-shogaol-mediated cell migration (C) and invasion (D) inhibition; overexpression of Snail decreases the up-regulation of 4-shogaol on E-cadherin and RKIP expression at mRNA (E) and protein (F) levels. MDA-MB-231 was transfected with pCMV or pSnail plasmid, and stable colonies were established by G418 selection. Snail-overexpressing cells were treated with 4-shogaol (7.5 μM) for the indicated times (24 h for E-cadherin and 6 h for RKIP), and various protein levels were assessed by immunoblot assay. The migration and invasion ability of MDA-MB-231 cells was determined by wound-healing assay and the transwell system. Each value is the mean ± SD of three determinations, and all results are representative of at least three independent experiments. The asterisk indicates a significant difference between the two test groups, as analyzed by Student's t test post hoc ($p < 0.05$).

4-Shogaol Decreases Metastasis of MDA-MB-231 Cells in Vivo. Animal experiments were carried out to determine whether 4-shogaol decreases lung metastasis of MDA-MB-231 in vivo. As shown in Figure 6A–D, lungs of mice treated with 4-shogaol contained fewer and smaller metastatic nodules than those of the control mice. The lung tissue invaded by tumor foci was characterized by high-grade proliferating tumor cells and numerous venous emboli. Moreover, the incidence of lung metastases was significantly lower in all 4-shogaol-treated mice. In addition, 4-shogaol also decreased the nuclei translocation of NF-κB and Snail in primary tumor sections (Figure 6E). 4-Shogaol increased the expression of RKIP in tumor sections in the lungs of MDA-MB-231-implanted mice (Figure 6F).

DISCUSSION

Breast cancer is the most common of all lethal malignancies and is also one of the four most prevalent malignant diseases of women in the world.[1,2] Current studies show that 4-shogaol, an active ingredient of ginger, decreased metastasis of MDA-MB-231 cells by decreasing cell migration and invasion. In addition, in vivo studies showed that ip injections of 4-shogaol significantly decrease the metastasis of MDA-MB-231 cells to the lungs of mice.

NF-κB has been reported to modulate the expression of several genes having products associated with tumor development.[28,29] Cancer cells in which NF-κB is constitutively active are highly metastatic, and inhibition of NF-κB activity in these cells greatly decreases their invasiveness.[30,31] The inflammatory condition of the cancer microenvironment has been reported to enhance NF-κB-mediated tumor growth and metastasis.[32,33] The activity of NF-κB is regulated by IκB, which sequesters NF-κB in the cytoplasm, resulting in the inhibition of NF-κB activity.[8] Signal-induced phosphorylation and ubiquitination of IκB result in proteosome-mediated degradation, which in turn causes nuclear translocation and DNA binding of NF-κB.[8] In this study, we have shown that treatment of MDA-MB-231 cells with 4-shogaol not only decreases nuclear translocation of NF-κB but also reduces its DNA-binding activity. This inhibitory effect of 4-shogaol on NF-κB is associated with a decrease of IκB phosphorylation and degradation. In addition, inflammatory cytokine TNF-α treatment increases the activation of NF-κB, which is, again, inhibited by 4-shogaol treatment. Furthermore, 4-shogaol also inhibits TNF-α-enhanced cancer migration and invasion. These data suggest that 4-shogaol is a potential inhibitor, targeting both constitutive and inducible activation of NF-κB.

Transcription factor Snail, one of the target genes of NF-κB, is a key regulatory factor in EMT and cell migration. Its expression is elevated in several cancer types, including breast cancer.[6,34] Snail transcriptionally suppresses the adherent junction protein, E-cadherin, by binding to E2-box type elements within its promoter, resulting in EMT.[35] E-cadherin loss and EMT have been implicated in the enhancement of metastatic ability and are closely correlated with poor prognosis.[36] In contrast, mesenchymal-type cancer cells can revert back to an epithelial phenotype via the MET process, thereby decreasing their metastatic capacity. In our study, we found that 4-shogaol has a significant inhibitory effect on Snail expression, which is consistent with the blockade of NF-κB by 4-shogaol. The inhibition of Snail is directly associated with restoration of E-cadherin and inhibition of mesenchymal gene markers (N-cadherin and vimetin). Overexpression of Snail decreases the antimigration properties and up-regulation of E-cadherin in MDA-MB-231 cells. Our findings also revealed that 4-shogaol decreases cancer metastasis by decreasing the location of Snail in cell nuclei in vivo. These results suggest that inhibition of Snail may be pivotal for 4-shogaol-mediated E-cadherin induction and promotion of MET.

RKIP, a metastatic suppressor, has been reported to inhibit NF-κB activity, directly inhibiting IKK activity by forming an inactive complex.[37,38] RKIP loss has been identified in cancers, whereas restoration of RKIP expression inhibits cancer

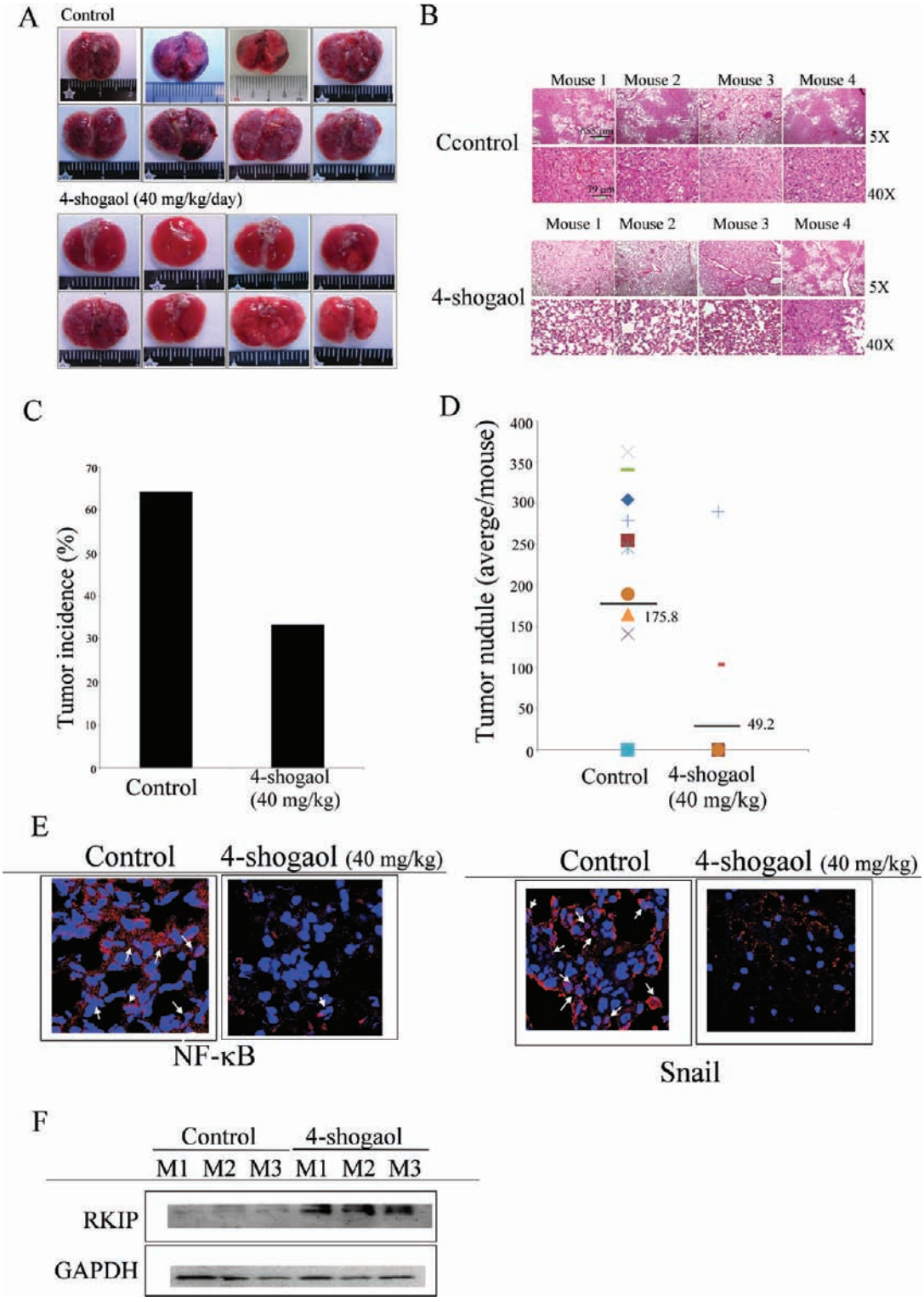

Figure 6. 4-Shogaol decreases metastasis of MDA-MB-231 cells in vivo: 4-shogaol decreases lung metastasis of MDA-MB-231 cells, as revealed by photographs (A) and H&E staining (B); (C) 4-shogaol decreases the incidence of MDA-MB-231 cell metastasis in mice; (D) 4-shogaol decreased tumor nodules in lungs; 4-shogaol decreases the nuclear translocation of NF-κB and Snail (E) and increases the expression of RKIP (F). Mice were injected with MDA-MB-231 cells via the tail vein and then randomly sorted into two groups. The mice in the 4-shogoal-treated group were ip injected daily with 4-shogaol in a clear solution containing 4% Cremophor EL (40 mg/kg of body weight), at a volume of 0.2 mL. After 35 days, nontumorous and tumorous regions of the lungs were harvested, cut, and stained and then analyzed by confocal microscopy (10× and 40×).

metastases in animal models.[39] Recent studies have indicated that RKIP expression is regulated by Snail. RKIP expression is inversely correlated with Snail expression in metastatic tumor samples, whereas overexpression of Snail decreases RKIP expression in cancers.[40,41] In this study, we have shown that 4-shogaol decreases the amount of Snail in the nuclei, subsequently relieving the repression of Snail on RKIP. This result supports the hypothesis that overexpression of Snail decreases

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the up-regulation of 4-shogaol on the up-regulation of RKIP. Reinforcement of RKIP by 4-shogaol further inhibits NF-κB by interacting with IKK to form an inactive complex, which in turn reduces IKK kinase activity, resulting in a decrease in IκB phosphorylation. Moreover, inhibition of RKIP by siRNA weakens the inhibitory effect of 4-shogaol on cell migration and invasion, suggesting that restoration of RKIP is a primary event in the antimetastatic property of 4-shogaol.

Our findings demonstrate that 4-shogaol inhibits both in vitro and in vivo breast cancer metastasis. 4-Shogaol inhibits the ability of MDA-MB-231 cells to migrate and invade by reducing the activation of NF-κB and Snail and sequentially increasing RKIP levels in these cells (Figure 7). Therefore, 4-shogaol is a potentially useful antimetastatic agent in the treatment of human breast carcinoma.

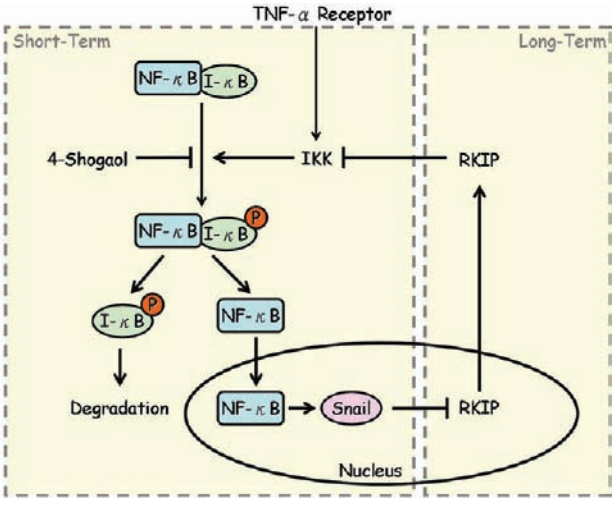

Figure 7. Molecular mechanism of 4-shogaol suppresses breast cancer metastasis. 4-Shogaol effectively inhibits the metastasis of breast cancer by decreasing NF-κB and Snail and sequentially results in the reinforcement of RKIP expression and the inhibition of cell migration and invasion.

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