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Timosaponin AIII inhibits melanoma cell migration by suppressing COX-2 and *in vivo* tumor metastasis

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Melanoma is the leading cause of death from skin disease, due in large part to its propensity to metastasize. We examined the effects of timosaponin AIII, a compound isolated from *Anemarrhena asphodeloides* Bunge, on melanoma cancer cell migration and the molecular mechanisms underlying these effects using B16-F10 and WM-115 melanoma cells lines. Overexpression of COX-2, its metabolite prostaglandin E_2 (PGE_2), and PGE_2 receptors (EP2 and EP4) promoted cell migration *in vitro*. Exposure to timosaponin AIII resulted in concentration-dependent inhibition of cell migration, which was associated with reduced levels of COX-2, PGE_2, and PGE_2 receptors. Transient transfection of COX-2 siRNA also inhibited cell migration. Exposure to 12-O-tetradecanoylphorbal-13-acetate enhanced cell migration, whereas timosaponin AIII inhibited 12-O-tetradecanoylphorbal-13-acetate-induced cell migration and reduced basal levels of EP2 and EP4. Moreover, timosaponin AIII inhibited activation of nuclear factor-kappa B (NF-κB), an upstream regulator of COX-2 in B16-F10 cells. Consistent with our *in vitro* findings, *in vivo* studies showed that timosaponin AIII treatment significantly reduced the total number of metastatic nodules in the mouse lung and improved histological alterations in B16-F10-injected C57BL/6 mice. In addition, C57BL/6 mice treated with timosaponin AIII showed reduced expression of COX-2 and NF-κB in the lung. Together, these results indicate that timosaponin AIII has the capacity to inhibit melanoma cell migration, an essential step in the process of metastasis, by inhibiting expression of COX-2, NF-κB, PGE_2, and PGE_2 receptors.

Melanoma is the most deadly type of skin cancer, accounting for approximately 80% of deaths caused by skin cancer.[1] Melanoma is particularly deadly due to its propensity to metastasize; clinical trials indicate that melanoma shows preferential metastasis to the lung, brain, liver, and skin. Moreover, melanoma is highly resistant to conventional chemotherapeutics.[2] Given the rising incidence of melanoma and the lack of effective therapies, development of new chemicals targeting the complex genetic networks involved in melanoma metastasis should provide new treatment strategies for this devastating disease.[3,4]

Two COX isoforms with distinct physiologic functions have been identified: COX-1 and COX-2. COX-1 is expressed constitutively in many tissues and has an important role in the maintenance of homeostasis. In contrast, COX-2 is an inducible enzyme that is activated by extracellular stimuli, such as UV radiation. Enhanced expression of COX-2 in skin exposed to UV radiation has been identified as a risk factor for the development of skin cancer.[5,6]

Cyclooxygenase-2 generates prostaglandins that are thought to play a central role in orchestrating the events involved in cancer invasion and metastasis. Prostaglandin E_2 (PGE_2) exerts its effects through G-protein coupled receptors (EP1, EP2, EP3, and EP4) and has been implicated in angiogenesis, invasion, and metastasis.[7,8] Because melanoma is a highly malignant cancer with the capacity to rapidly metastasize to distant sites, approaches inhibiting metastasis and/or migration of melanoma cells may facilitate the development of effective treatment and prevention strategies.

Timosaponin AIII is isolated from the medicinal herb *Anemarrhena asphodeloides* Bunge, which is used as an antipyretic, anti-inflammatory, antidiabetic, and antidepressive agent in traditional Asian medicine.[9,10] Experimental evidence shows that purified timosaponins and fractions of *A. asphodeloides* extracts containing timosaponins show various pharmacological properties, including improvement of learning and memory in subjects with dementia.[9,10] Recently, timosaponin AIII was also shown to be preferentially toxic to breast cancer cell lines over non-transformed cells.[11] Therefore, we assessed the effects of timosaponin AIII on the migration potential of melanoma cells using *in vitro* assays and an *in vivo* metastasis model in mice, in which timosaponin AIII had not previously been evaluated. In this study, we assessed the chemotherapeutic effects of timosaponin AIII by evaluating melanoma cell migration, because tumor cell migration is a major event in the metastatic cascade. We also explored the involvement of COX-2, nuclear factor-κB (NF-κB), PGE_2, and PGE_2 receptors in melanoma cell migration.

Materials and Methods

Chemicals. Timosaponin AIII was isolated from *A. asphodeloides* as previously described.[12] 12-O-tetradecanoylphorbal-13-acetate (TPA) and PGE_2 were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Antibodies against COX-2, EP2, EP4, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against NF-κB, IκB kinase α (IKKα), and inhibitor of NF-κB α (IκBα) were obtained from Cell Signaling Technology (Beverly, MA, USA). PGE_2 immunoassay kits were obtained Cayman Chemical (Ann Arbor, MI, USA).

Cell culture. B16-F10 murine melanoma cells and WM-115 human melanoma cells were purchased from the ATCC (Manassas, VA, USA). B16-F10 cells were grown to confluence in DMEM with 10% FBS and 1% penicillin/streptomycin. WM-115 cells were cultured in Eagle's minimum essential medium containing 10% FBS, 2 mM glutamine, 1% non-essential amino acids, and 1% sodium pyruvate at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Cell viability. B16-F10 and WM-115 cells (1×10^4) were seeded in 96-well culture plates in the presence or absence of timosaponin AIII. After 24 h, cell viability was assessed by incubation with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) for 1 h and measuring its reduction to formazan, according to the manufacturer's instructions; samples were assayed at 490 nm using a microplate fluorimeter (Molecular Devices, Sunnyvale, CA, USA).

Migration assay. The chemotactic motility of B16-F10 and WM-115 cells were assayed using Transwell chambers (Corning Costar, Cambridge, MA, USA) with 6.5-mm diameter polycarbonate filters (8-μm pore size). The lower surface of each filter was coated with 10 μg gelatin. Fresh DMEM (with 1% FBS) was placed in the lower wells. Cells were trypsinized and suspended at a final concentration of 1×10^5 cells/mL in DMEM containing 1% FBS, followed by treatment with the indicated concentrations of timosaponin AIII at room temperature for 30 min prior to seeding. The cell suspension (100 μL/well) was loaded into the upper wells and the chambers were incubated for 24 h at 37°C, after which the cells were fixed and stained with H&E. Non-migrating cells on the upper surface of each filter were removed with a cotton swab. Chemotaxis was quantified by counting the cells that had migrated to the lower side of the filter with an optical microscope (magnification, ×100). Five fields were counted per assay.

Prostaglandin E_2 immunoassay for quantitation of PGE_2. Analysis of PGE_2 in the cell homogenates was carried out using the Cayman PGE_2 Enzyme Immunoassay Kit following the manufacturer's instructions. Briefly, cells were harvested at the indicated time points and homogenized in 100 mM phosphate buffer (pH 7.4) containing 1 mM EDTA and protease inhibitors using a homogenizer. Homogenates were centrifuged and the resulting supernatants were collected and subjected to PGE_2 measurement according to the manufacturer's instructions.

Cycloogenase-2 siRNA transfection of B16-F10 and WM-115 cells. Human and mouse COX-2 siRNAs were purchased from Santa Cruz Biotechnology. Each siRNA (mixture of 50 nM COX-2 and 50 nM control siRNA) was transfected into cells using Lipofectamine reagent according to the manufacturer's protocol. The transfected cells were cultured in DMEM. WM-115 cells were cultured in Eagle's minimum essential medium without antibiotics for 8 h, cultured in complete medium for 36 h, harvested, and subjected to the cell migration assay. Knockdown of COX-2 expression in cells after transfection was confirmed using Western blot analysis.

Wound healing assay. B16-F10 cells (5×10^4 cells/well) and WM-115 cells (5×10^4 cells/well) were seeded in six-well plates and incubated for 24 h. Cells monolayers were wounded with a sterile 100-μL pipette tip and washed with growth medium to remove detached cells from the plates. Cells were exposed to various concentrations of TPA and incubated for 24 h. After 24 h, the medium was replaced with PBS and the cells were photographed using an Olympus BX41 microscope (Tokyo, Japan) and a digital camera. To quantify cell migration, images of the initial wounded monolayers were compared to corresponding pictures of the cells at later time points.

Preparation of cytosolic and nuclear fractions for Western blot analysis. Cytosolic and nuclear fractions were prepared from B16-F10 cells according to the manufacturer's instructions (Paris Kit; Life Technologies, Carlsbad, CA, USA). Briefly, cells were treated with timosaponin AIII (or left untreated) and harvested. Cells were trypsinized, suspended in cell fractionation buffer, loosened by flicking the tube, and incubated on ice for 5 min. The suspension was centrifuged at 500 *g* for 5 min at 4°C and the supernatant containing the cytoplasmic cell fraction was collected. The pellet containing the nuclear fraction was resuspended with ice-cold cell disruption buffer, vortexed, and incubated for 5 min on ice to ensure complete cell disruption. Nuclear NF-κB was detected by Western blotting.

Western blot analysis. Cell lysates were prepared by lysing B16-F10 cells (5×10^5 cell/well) in 1× Laemmli lysis buffer (2.4 M glycerol, 0.14 M Tris [pH 6.8], 0.21 M SDS, and 0.3 mM bromophenol blue) followed by boiling for 10 min. Protein content was measured using the BCA Protein Assay Reagent (Pierce, Rockfor, IL, USA). Each sample (20 μg) was diluted with 1× lysis buffer and proteins were separated by SDS-PAGE on 4.5–15% gradient gels. The proteins were transferred onto PVDF membranes, which were incubated with primary antibodies against COX-2, NF-κB, IKKα, IκBα, EP2, and EP4. Specifically bound HRP-conjugated secondary antibodies were detected using an ECL detection system (Amersham Bioscience, Little Chalfont, UK). Protein expression levels were determined by analyzing the signals captured on the PVDF membranes using an image analyzer (Las-3000; Fujifilm, Tokyo, Japan).

Nuclear factor-κB/p65 activity assay. For quantitative analysis of NF-κB/p65 activity, the NF-κB TransAM Activity Assay kit (Active Motif, Carlsbad, CA, USA) was used following the manufacturer's protocol and as carried out previously.[13]

***In vivo* lung metastasis assay.** C57BL/6 mice were used in this study. Animal experiments and care were carried out according to the National Research Council Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Daejeon University (DJUARB201-038) (Daejeon, Korea). B16-F10 melanoma cells were harvested with Trypsin solution and resuspended at appropriate concentrations in PBS. Cells were injected into the lateral tail vein of mice. The mice were randomly assigned to four groups (nine mice per group): I, normal control group; II, melanoma cell-injected (5×10^4 cells/mouse) and untreated; III, melanoma cell-injected and treated with 12.5 mg/kg timosaponin AIII; and IV, melanoma cell-injected mice treated with 25 mg/kg timosaponin AIII. One hour after injection of the B16-F10 melanoma tumor cells, the mice were treated with a single dose of timosaponin AIII (12.5

or 25 mg/kg) by the i.p. route. Fourteen days later, the mice were killed by cervical dislocation and the lungs were removed, washed with PBS, and fixed with Bouin's solution for 24 h to facilitate counting of tumor nodules as previously described.[14] The number of surface tumor nodules was counted under a dissecting microscope. Sections of the lungs were stained with H&E to confirm that the nodules were malignant and to monitor the presence of lung fibrosis.

Histopathological analysis of the lung. Lung tissue (tumor nodules) was collected after the mice were killed. The tissue samples were fixed in 10% formaldehyde, dehydrated, and embedded in paraffin wax for histological studies. Tissue sections (5 μm) were stained with H&E, mounted in DPX, and examined under a microscope to detect histopathological evidence of lung cancer.[15]

Isolation of RNA. For preparation of total RNA, lung tissue and B16-F10 cells were homogenized in TRIzol according to the manufacturer's instructions, followed by RNA extraction with chloroform. Total RNA samples were quantified and stored at −20°C. Aliquots of total RNA (2 μg) were used for the cDNA synthesis.

Reverse transcription PCR. To quantify gene expression in lung tissue, RT-PCR analysis was carried out (ABI QuantStudio 6 Flex Real-Time PCR system; Applied Biosystems, Waltham, MA, USA) using *Taq*Man universal PCR Master Mix (Applied Biosystems). The relative mRNA expression levels of COX-2 and NF-κB were normalized to that of hypoxanthine phosphoribosyltransferase in lung tissue; mRNA expression levels of COX-2 was normalized to that of β-actin. The PCR protocol consisted of 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was assayed in triplicate and relative mRNA expression levels were calculated using the $^{\Delta\Delta}$Ct method.

Statistical analysis. Data are presented as the mean ± SD of at least three separate experiments. Comparisons between three groups were carried out with Student's *t*-test in Prism software (GraphPad Software, San Diego, CA, USA). The significance threshold was $P < 0.05$.

Results

Timosaponin AIII inhibits melanoma cell migration and COX-2 expression. The chemical structure of timosaponin AIII is shown in Figure 1(a). Twenty-four hours of treatment with various concentrations of timosaponin AIII caused no detectable cytotoxicity, measured by MTS assay (data not shown). We first determined whether treatment of mouse melanoma cells (B16-F10) and human melanoma cells (WM-115) with timosaponin AIII inhibited their migration using the Boyden chamber cell migration assay. As shown in Figure 1(b), relative to untreated control cells, treatment with timosaponin AIII at concentrations of 10, 50, and 100 nM for 24 h reduced the migratory capacity of B16-F10 and WM-115 cells in a concentration-dependent manner. The numbers of migrating cells per microscopic field are summarized in Figure 1(c,d). Next, experiments were carried out to determine whether the inhibitory effect of timosaponin AIII on cell migration was associated with inhibition of basal COX-2 expression. As shown in Figure 1(e), treatment of B16-F10 cells and WM-115 cells with timosaponin AIII reduced COX-2 expression in a concentration- and time-dependent manner in comparison with that of control cells, suggesting that timosaponin AIII exerted an inhibitory effect on melanoma cell migration that was associated with reduced COX-2 expression.

Inhibition of melanoma cell migration by timosaponin AIII is associated with inhibition of endogenous COX-2 expression and PGE_2 production. We next carried out experiments to determine whether the inhibitory effects of timosaponin AIII on melanoma cell migration were mediated through its inhibitory effect on COX-2 expression. Treatment with timosaponin AIII for 24 h dose-dependently decreased PGE_2 production in

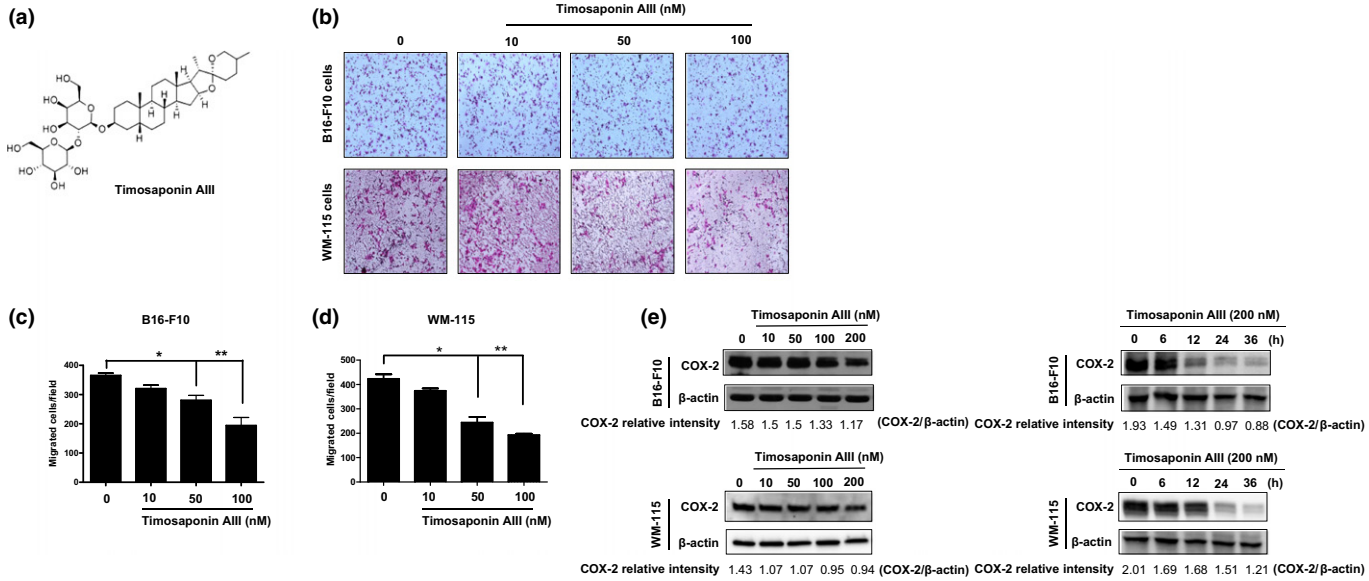

Fig. 1. Timosaponin AIII inhibits melanoma cell migration and COX-2 expression. (a) Structure of timosaponin AIII. (b) Treatment of melanoma cells with timosaponin AIII for 24 h prior to 30 min inhibited migration of B16-F10 and WM-115 cells in a concentration-dependent manner. (c, d) Migrating B16-F10 and WM-115 cells were counted and the results were expressed as the mean number of migratory cells ± SD per microscopic field. (e) Effect of timosaponin AIII on the endogenous basal level of COX-2 in B16-F10 and WM-115 cells. COX-2 protein levels were determined by Western blot analysis. *$P < 0.01$, **$P < 0.005$, timosaponin A III-treated group *versus* non-timosaponin A III-treated control group.

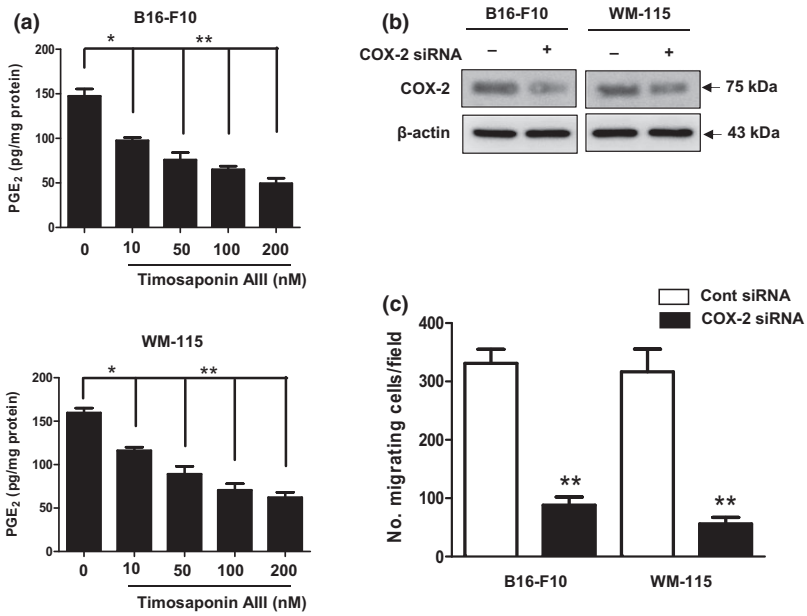

Fig. 2. Timosaponin AIII inhibits melanoma cell migration, endogenous COX-2 expression, and prostaglandin E_2 (PGE_2) production. (a) Dose-dependent effect of timosaponin AIII on PGE_2 expression in B16-F10 and WM-115 melanoma cells. PGE_2 levels are expressed in term of pg/mg protein ± SD (three independent experiments). $^*P < 0.01$, $^{**}P < 0.005$, timosaponin A III-treated group *versus* non-timosaponin A III-treated control group. (b) Transfection of B16-F10 and WM-115 cells with COX-2 siRNA markedly reduced COX-2 protein levels. (c) Transfection of cells. COX-2 siRNA significantly inhibited migration of B16-F10 and WM-115 cells. B16-F10 and WM-115 cells were transfected with COX-2 siRNA to knockdown COX-2 expression. $^{**}P < 0.005$, significant reduction of cell migration *versus* control (Cont) siRNA-treated cells.

B16-F10 and WM-115 cells (Fig. 2a). We further verified the role of COX-2 in cell migration by determining whether siRNA knockdown of COX-2 in melanoma cells inhibited cell migration. Using Western blot analysis, we confirmed that transfection of COX-2 siRNA into B16-F10 and WM-115 cells significantly reduced COX-2 expression in comparison with

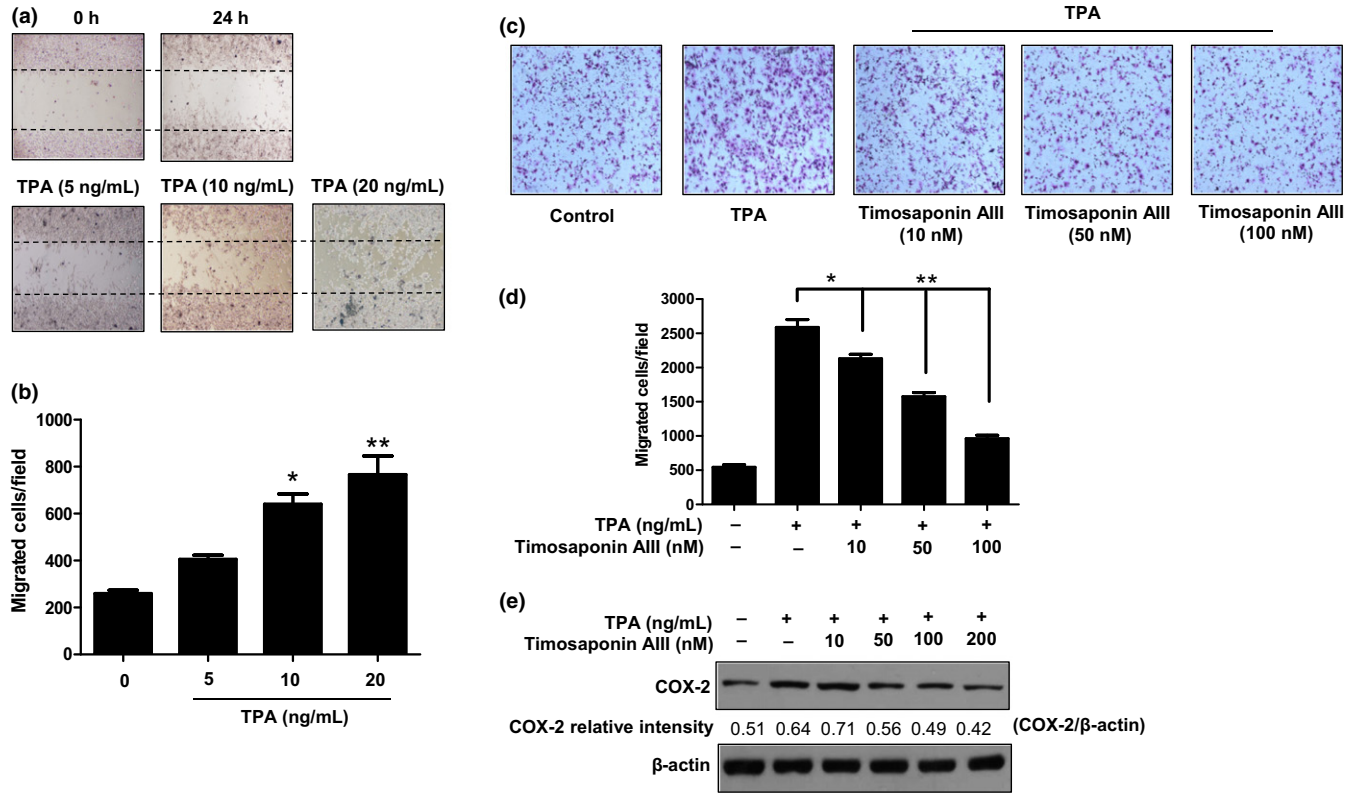

Fig. 3. Timosaponin AIII inhibits TPA-induced melanoma cell migration. (a) Treatment of B16-F10 cells with 12-O-tetradecanoylphorbal-13-acetate (TPA), a COX-2 stimulator, enhanced cell migration in a concentration-dependent manner. (b) Migrating B16-F10 cells were counted and the results were expressed as the mean number of migratory cells ± SD per microscopic field. $^*P < 0.01$, $^{**}P < 0.005$ significantly increased migration *versus* control cells. (c,d) Treatment of B16-F10 cells with timosaponin AIII (10, 50, or 100 nM) inhibited TPA-enhanced cell migration. $^*P < 0.01$, $^{**}P < 0.005$, significant inhibition *versus* TPA alone. (e) Timosaponin AIII downregulated TPA-induced COX-2 expression in B16-F10 cells. COX-2 protein levels were determined in cell lysates using Western blot analysis.

transfection of the control siRNA (Fig. 2b). Transfection of B16-F10 and WM-115 cells with COX-2 siRNA significantly reduced migration of both cell types (Fig. 2c). As the inhibitory effects of timosaponin AIII on migration of B16-F10 and WM-115 cells were very similar, subsequent studies were carried out using B16-F10 cells.

Timosaponin AIII inhibits TPA-induced migration of melanoma cells. Treatment of skin with TPA stimulates COX-2 expression.[16,17] Therefore, we determined the effects of TPA on melanoma cell migration. As shown in Figure 3(a), treatment of B16-F10 cells with TPA for 24 h significantly enhanced cell migration in a dose-dependent manner. Similar resulted were noted for the numbers of migrating cells per microscopic field (Fig. 3b). To determine whether timosaponin AIII inhibit TPA-induced cell migration in melanoma cells, B16-F10 cells were treated with TPA (20 ng/mL) with or without timosaponin AIII for 24 h. Treatment of B16-F10 cells with timosaponin AIII dose-dependently inhibited TPA-induced cell migration (Fig. 3c,d). To verify whether inhibition of cell migration by timosaponin AIII was mediated through inhibition of TPA-induced COX-2 expression, we treated B16-F10 cells with TPA with or without timosaponin AIII for 24 h, showing that timosaponin AIII abrogated COX-2 expression in a concentration-dependent manner (Fig. 3e). These results suggest that timosaponin AIII inhibits TPA-induced cell migration by downregulating COX-2 expression.

Timosaponin AIII decreases abundance of PGE_2 receptor and NF-κB/p65 activity in B16-F10 melanoma cells. Prostaglandin E_2 produces its biological activity through four G-protein-coupled receptors (EP1–EP4).[18] We determined the effects of timosaponin AIII on basal levels of PGE_2 receptors in B16-F10 melanoma cells. As shown in Figure 4a, exposure to timosaponin AIII (10, 50, 100, and 200 nM) for 24 h dose-dependently reduced protein levels of EP2 and EP4. Similar inhibitory effects on EP1 and EP3 protein levels were observed, but these effects were less prominent than the effects of timosaponin AIII on EP2 and EP4 (data not shown). Next, we examined whether timosaponin AIII inhibited induction of EP2 and EP4 expression by PGE_2 in B16-F10 melanoma cells. The cells were treated with PGE_2 (10 μg/mL) for 24 h with or without timosaponin AIII. Prostaglandin E_2 treatment increased levels of EP2 and EP4, but this effect was inhibited by timosaponin AIII (Fig. 4b). Because COX-2 is a downstream target gene of NF-κB, we assessed whether timosaponin AIII altered expression of NF-κB in B16-F10 melanoma cells. Timosaponin AIII treatment reduced translocation of NF-κB/p65 from the cytosol to the nucleus in a dose-dependent manner (Fig. 4c). Moreover, NF-κB activity was significantly reduced by timosaponin AIII treatment in a concentration-dependent manner (Fig. 4d). In addition, timosaponin AIII treatment resulted in downregulation of IKK-α and degradation of IκBα (Fig. 4c), which would be expected to inactivate NF-κB and inhibit its translocation into the nucleus. Also, timosaponin AIII treatment resulted in downregulation of COX-2 in a concentration-dependent manner in B16-F10 cells (Fig. 4e).

Timosaponin AIII inhibits metastasis of melanoma cells to the lung in mice. Taking into account the results obtained *in vitro*, we examined whether the antimetastatic effect of timosaponin AIII against B16-F10 melanoma cells was produced *in vivo*.

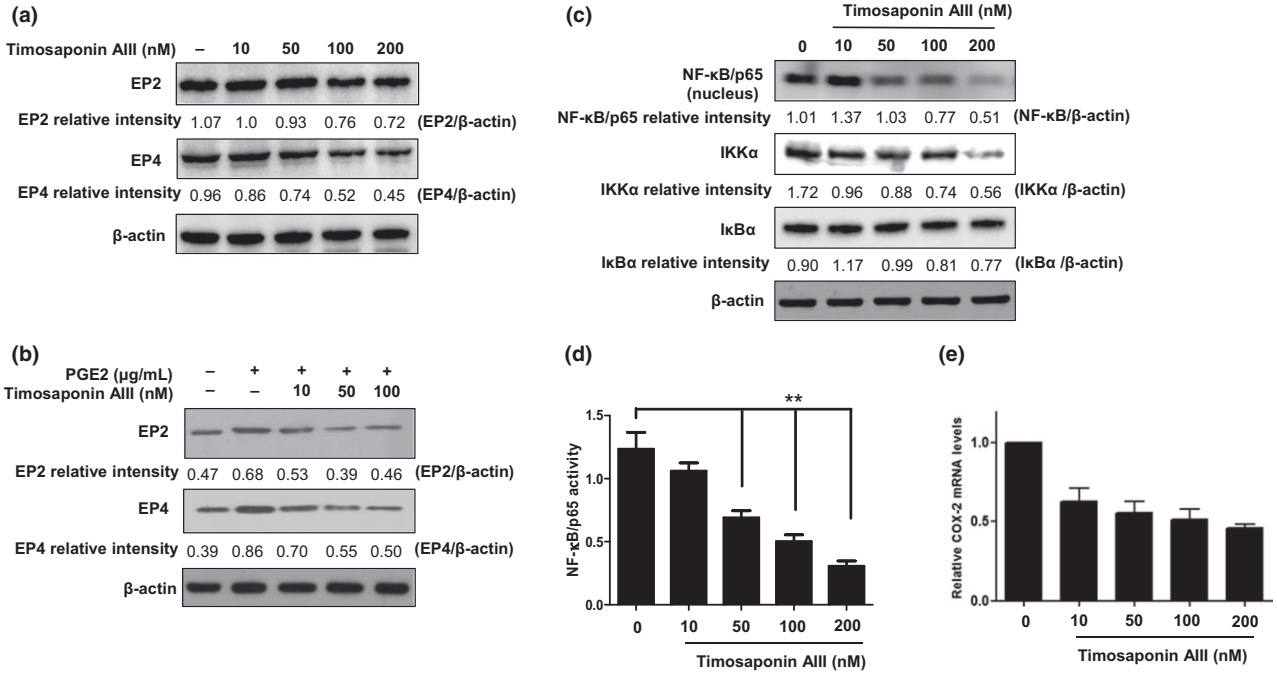

Fig. 4. Timosaponin AIII decreases prostaglandin E_2 (PGE_2) receptor levels and nuclear factor-κB (NF-κB) activity in B16-F10 cells. (a) Treatment of B16-F10 cells with timosaponin AIII for 24 h decreased expression levels of PGE_2 receptors EP2 and EP4 in a concentration-dependent manner. Cell lysates were prepared and subjected to Western blot analysis. (b) Timosaponin AIII decreased PGE_2-enhanced expression of EP2 and EP4. Cell lysates were prepared and subjected to Western blot analysis. (c) After treatment with various concentrations of timosaponin AIII for 24 h, cells were harvested and cytosolic and nuclear fractions were prepared. NF-κB, IκB kinase α (IKKα), and inhibitor of NF-κB α (IκBα) protein levels were measured using Western blot analysis. (d) NF-κB activity in the nuclear fractions after treatment with or without timosaponin AIII for 24 h was measured using an NF-κB/p65-specific activity assay kit. NF-κB activity is expressed in terms of that of the control cells (%). (e) Treatment of B16-F10 cells with timosaponin AIII for 24 h decreased expression levels of COX-2 in a concentration-dependent manner. The relative mRNA expression levels of COX-2 was normalized to that of β-actin, which served as an internal control. $^{**}P < 0.005$, significant decrease *versus* control group.

Injection of B16-F10 cells into the lateral tail vein induced metastasis to the lung in mice. Two weeks of timosaponin AIII treatment (12.5 or 25 mg/kg) reduced the number of nodules per mouse. This observation was confirmed by statistical analysis of the number of tumor nodules, which showed that the difference between the B16-F10 cell-treated group and the timosaponin AIII-treated group was significant (Fig. 5a). Moreover, timosaponin AIII treatment markedly reduced mRNA levels of COX-2 and NF-κB in the lung (Fig. 5b). Histopathology of the lung also showed a marked reduction in the tumor mass in the lungs of the timosaponin AIII-treated animals (Fig. 5c). These results indicate that timosaponin AIII exerts antimetastatic activity *in vivo*.

Discussion

Metastasis is the process by which a certain cancer spreads from the location at which a tumor first arises to distant locations in the body.[19] Metastasis is usually dependent on cancer cells having motility and invasive capacity. Recurrence of cancer through metastasis is one of the main causes of mortality in cancer patients and a main target for cancer therapy.[20] Therefore, innovative strategies are required to prevent melanoma cell migration and invasion.

Many human cancers express elevated levels of COX-2, an enzyme responsible for the biosynthesis of PGs. Overexpression of COX-2 and abundant production of PGs, and particularly PGE_2, have been linked with tumor progression, invasion, and metastasis.[21,22] Because of its important role in tumor invasion and metastasis, COX-2 is a promising target of cancer therapeutics.[8,23] The COX metabolite, PGE_2, in turn exerts its effects though G protein-coupled receptors EP1–4. Prostaglandin E_2, produced at heightened levels in COX-2 overexpressing tumor cells, affects target cells through interaction with EP receptors of four distinct subtypes.[24] Therefore, the development of potential COX-2 and PGE_2 inhibitors to prevent and

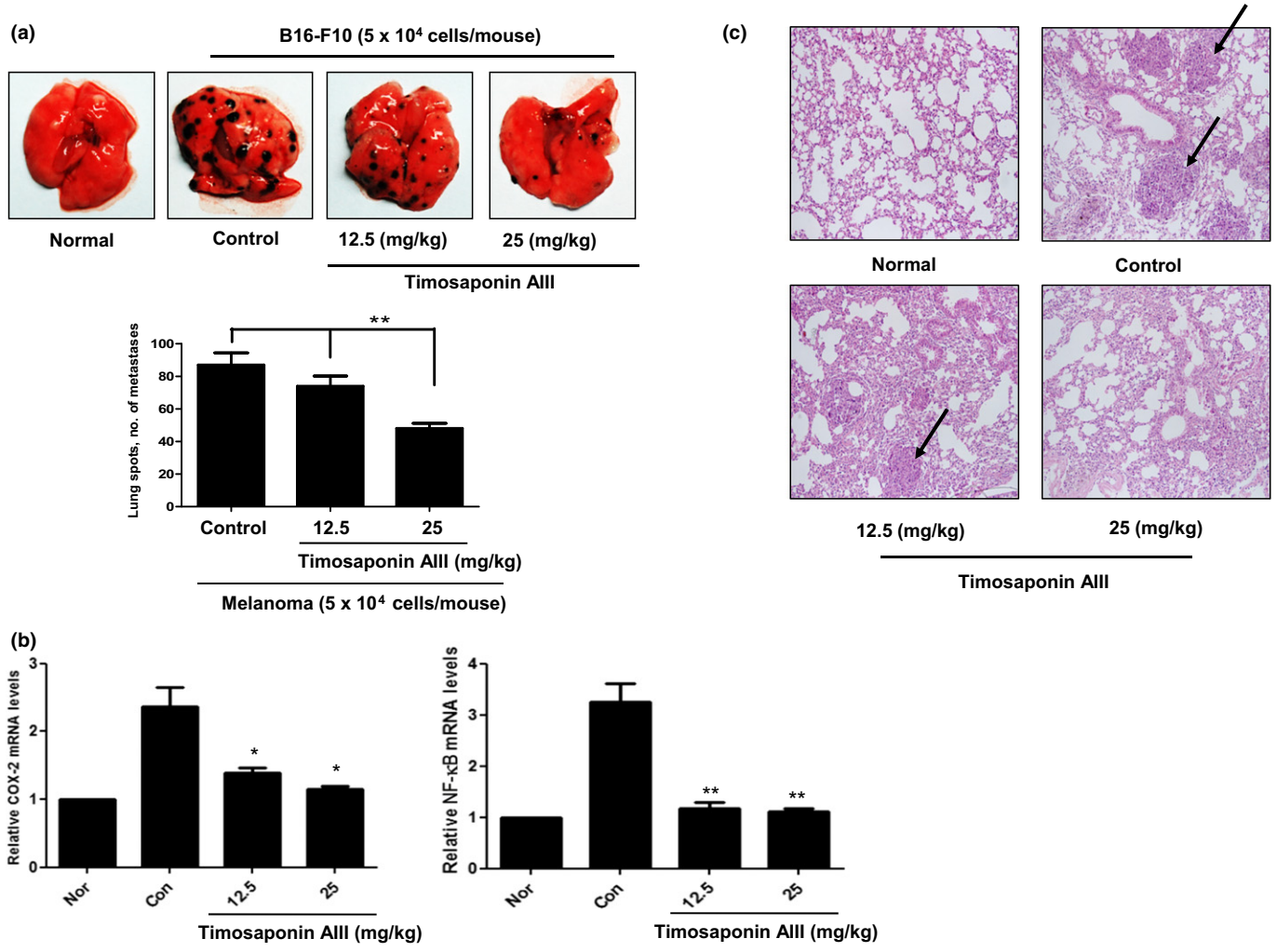

Fig. 5. Timosaponin AIII reduces metastasis of B16-F10 melanoma cells to lung in mice. (a) B16-F10 cells (5×10^4 cells/0.2 μL) were injected into the lateral tail vein of C57BL/6 mice, after which they received a single dose of timosaponin AIII (12.5 or 25 mg/kg) by the i.p. route. After 14 days, visible nodules of metastasized tumors in the lung were quantified. Results are expressed as mean ± SD ($n = 9$). $^{**}P < 0.005$ in comparison with melanoma-induced mice. (b) To quantify COX-2 and NF-κB gene expression in the lung, real-time quantitative PCR analysis was carried out. The relative mRNA expression levels of COX-2 and NF-κB in the lung were normalized to that of hypoxanthine phosphoribosyltransferase, which served as an internal control (Con). Data are represented as mean ± SD ($n = 3$). $^{**}P < 0.01$, $^{*}P < 0.05$, *versus* control group. (Con),. (c) H&E staining of 5-μm paraffin-embedded lung tissue sections from each group. Arrows show lung-infiltrating tumor cells. Original magnification, ×100 Nor: normal group.

treat melanoma may improve cancer treatment strategies. The most significant finding of the present study is that treatment of melanoma cells with timosaponin AIII for 24 h inhibits cell migration in a dose-dependent manner that is associated with inhibition of COX-2 expression and PGE_2 production. Moreover, melanoma cells overexpress COX-2; therefore, inhibition of COX-2 expression by timosaponin AIII may contribute to its inhibitory effect on migration of melanoma cells.

Effects similar to those of timosaponin AIII were observed when B16-F10 and WM-115 melanoma cells were transfected with COX-2 siRNA. It has been shown that TPA activates COX-2 expression; we found that treatment of melanoma cells with TPA enhanced cell migration, and this effect was blocked by timosaponin AIII. These results support the finding that inhibition of melanoma cell migration by timosaponin AIII requires inhibition of COX-2 expression. Prostaglandin E_2 exerts its biological functions through four G-protein coupled receptors, EP1–4,[8,18] activation of which stimulates epithelial cell growth, invasion, and cellular survival.[25,26] Prostaglandin E_2 promotes lung cancer cell migration through activation of PGE_2 receptors.[27] Because PGE_2 is a major prostaglandin associated with skin tumor promotion, progression, and invasion,[21] we assessed the involvement of PGE_2 receptors in timosaponin AIII-induced inhibition of melanoma cell migration. We observed that B16-F10 melanoma cells overexpressed PGE_2 receptors EP2 and EP4, expression of which was decreased when cells were treated with timosaponin AIII (Fig. 4a,b).

These data suggest that inhibition of EP2 and EP4 expression by timosaponin AIII may contribute to its inhibitory effect on cell migration. Furthermore, our findings indicate the feasibility of using timosaponin AIII as an alternative to COX-2 inhibitors, which show toxicity in some patients. Similar to timosaponin AIII, other phytochemicals have been examined for their effects on cell migration.[28] It has been reported that treatment of non-small-cell lung cancer cells with proanthocyanidins inhibits cell migration through inhibition of the nitric oxide and guanylate cyclase pathway. Another dietary polyphenol, (−)-epigallocatechin-3-gallate from green tea, inhibits mammary cancer cell migration by inhibiting nitric oxide and nitric oxide-mediated mechanisms.[28]

Because COX-2 is a downstream target of the NF-κB pathway, we assessed the effect of timosaponin AIII on basal levels of NF-κB in melanoma cells. Treatment of melanoma cells with timosaponin AIII downregulated basal levels of NF-κB protein in a dose-dependent manner (Fig. 4c,d). Also, timosaponin AIII treatment resulted in downregulation of COX-2 in a concentration-dependent manner in B16-F10 cells (Fig. 4e). These results suggest that the inhibitory effects of timosaponin AIII on melanoma cell migration is mediated, at least in part, through downregulation of COX-2, PGE_2, and PGE_2 receptor expression. However, it remains possible that downregulation of other NF-κB target genes could also contribute to the inhibitory effect of timosaponin AIII on cell migration.

The results described above clearly show the antimetastatic effect of timosaponin AIII *in vitro*. An *in vivo* study was carried out to demonstrate the antimetastatic effect of timosaponin AIII by counting tumor nodules in the lungs of C57BL/6 mice and histopathological analysis. Timosaponin AIII significantly inhibited formation of tumor nodules in the lung. Histopathological analysis confirmed that B16-F10-induced melanoma cells had a high metastatic potency to the host lung. B16-F10 metastasis effect was reversed by timosaponin AIII treatment, which reduced the massive tumor cell proliferation around the bronchioles of the lung (Fig. 5). Our results have established for the first time that timosaponin AIII inhibits migration of melanoma cells by inhibiting endogenous COX-2 overexpression and downregulating PGE_2 and PGE_2 receptors. Moreover, timosaponin AIII also inhibits formation of tumor nodules and improves histological parameters in the lungs of metastatic tumor-bearing mice.

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Disclosure Statement

The authors have no conflict of interest.

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