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Kalopanaxsaponin A Inhibits the Invasion of Human Oral Squamous Cell Carcinoma by Reducing Metalloproteinase-9 mRNA Stability and Protein Trafficking

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An inability to control cancer cell invasion and metastasis is the leading cause of death in patients with cancer. The present study was performed to determine the anti-invasive effect of Kalopanaxsaponin A (KPS-A) on matrix metalloproteinase-9 (MMP-9)-meidated invasion in phorbol 12-myristate 13-acetate (PMA)-stimulated human oral squamous cell carcinoma (OSCC) cells and a murine xenograft model of human OSCC. KPS-A, isolated from *Kalopanax pictus*, inhibited PMA-induced proliferation and invasion as well as PMA-induced MMP-9 expression and secretion at non-cytotoxic doses. KPS-A treatment reduced the stability of PMA-induced MMP-9 mRNA and inhibited the PMA-induced cytoplasmic translocation of HuR. In PMA-treated cells, KPS-A treatment resulted in the intracellular accumulation of MMP-9 and suppressed Ras-associated binding 1A (Rab1A) expression. KPS-A treatment suppressed PMA-induced phosphorylation of extracellular signal regulated kinase (ERK)1/2 and Akt. Furthermore, the oral administration of KPS-A led to substantial inhibition of tumor growth and the expression of proliferating cell nuclear antigen (PCNA), MMP-9, tissue inhibitor of metalloproteinase-1 (TIMP-1), HuR, and Rab1A in the tumor tissues of mice inoculated with YD-10B OSCC cells. Collectively, KPS-A inhibits the invasiveness of oral cancer by reducing HuR-mediated MMP-9 mRNA stability and Rab1A-mediated MMP-9 secretion *via* ERK1/2 and phosphatidylinositide 3-kinase (PI3K)/Akt. Therefore, KPS-A is a promising anti-invasive agent.

Key words Kalopanaxsaponin-A; oral cancer; anti-invasive effect; metalloproteinase-9; HuR; Ras-associated binding 1A

Oral squamous cell carcinoma (OSCC) is a common malignant tumor in the oral and maxillofacial region, and it is the sixth most common cancer worldwide. In high-risk countries such as Sri Lanka, India, Pakistan, and Bangladesh, oral cancer may contribute up to 25% of all new cases of cancer, and in most countries, the five-year survival rates for cancers of the tongue, oral cavity and oropharynx are around 50%.[1] OSCC is characterized by a high degree of local invasiveness and a high rate of metastasis to the cervical lymph nodes.[2] Despite multidisciplinary treatment with surgery, chemotherapy and radiation, the overall survival rate has not improved significantly in patients with oral cancer. In addition, many patients who are successfully treated for oral cancer have to cope with devastating consequences in their appearance and function, including eating, drinking, swallowing and speaking.[1] Consequently, controlling the invasiveness and metastasis of oral cancer represents an important therapeutic goal.

OSCC progression is frequently associated with the acquisition of a more invasive phenotype and with proteinase-dependent extracellular matrix (ECM) degradation.[2] Invasive OSCC cells secrete matrix metalloproteinases (MMPs), which are mainly responsible for the degradation of environmental barriers such as ECM and the basement membrane.[3,4] Although several other MMPs may also contribute to OSCC invasion,[5—7] MMP-9 (also known as gelatinase-B and 92-kDa type IV collagenase) has been shown to be involved in the oral cancer invasion process. In OSCC, MMP-9 expression has been reported to be induced by tumor necrosis factor-alpha,[8] epidermal growth factor,[9] and transforming growth factor-beta1.[10] Moreover, increased MMP-9 expression has been observed in invasive and metastatic cases of human OSCC.[11,12] Thus, MMP-9 can be used to identify the metastatic phenotype and to monitor treatment in oral cancer patients,[13,14] and downregulation of MMP-9 expression and activity may have therapeutic potential for preventing oral cancer invasion and metastasis.

Kalopanaxsaponin A (KPS-A; 3-*O*-[L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl]hederagenin) is an oleanane triterpene saponin found in *Kalopanax pictus* NAKAI (Araliaceae), a plant that has been traditionally used for the treatment of rheumatoidal arthritis and diabetes mellitus in East Asian countries.[15] KPS-A has been shown to reduce the levels of inducible nitric oxide synthase and cyclooxygenase-2 in lipopolysaccharide-treated RAW 264.7 cells,[16] to have a potent antimutagenic effect against aflatoxin B1 as well as cytotoxic effects in several types of cancer cells,[17] to inhibit the growth of colon and lung carcinomas in mice,[18,19] and to induce apoptosis through Ca^{2+} influx and caspase-8 dependent signaling pathways in human leukemia U937 cells.[20]

In this study, we examined the effects of KPS-A on phorbol 12-myristate 13-acetate (PMA)-induced invasiveness and MMP-9 expression in human OSCC cells. PMA, a well-known inflammatory stimulator and tumor promoter, has been known to activate protein kinase C and to stimulate cell invasion by activating MMP-9 *via* the distinct signaling pathway in various types of cancer cells.[21—23] KPS-A inhibited PMA-induced MMP-9 mRNA stability and protein secretion by controlling PMA-induced expressions of the mRNA binding protein HuR and the membrane trafficking protein Ras-associated binding (Rab)1A *via* both extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K)/Akt, thus preventing cell invasion. Moreover, this is the first paper demonstrating the roles of HuR and Rab1A in MMP-9-mediated invasion of oral squamous cell carcinoma, and reinforcing the clinical

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observations that HuR and Rab1A protein levels may be correlated with poor prognosis of oral cancer patients.[24,25]

MATERIALS AND METHODS

Cell Culture, Antibodies and Reagents Highly invasive YD-10B human OSCC cells, which were derived from a patient's tongue cancer tissues and previously characterized,[26] were cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS), 1×10^{-10} M cholera toxin, 0.4 mg/mL hydrocortisone, 5 μg/mL insulin, 5 μg/mL transferrin and 2×10^{-11} M triiodothyronine (T3) as a complete medium in a humidified atmosphere of 5% CO_2 at 37°C. The following antibodies were purchased from their respective sources: p38 and phospho-p38 mitogen activating protein kinase (MAPK) (New England Biolabs, Beverly, MA, U.S.A.); Akt, phospho-Akt, ERK1/2, and c-jun N-terminal kinase (JNK) (Cell Signaling Technology, Danvers, MA, U.S.A.); MMP-9, p50, and p65, tissue inhibitor of metalloproteinase (TIMP)-1, Ras-associated binding (Rab)1A (C-19), and HuR (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.); β-actin (Sigma Chemical, St. Louis, MO, U.S.A.); proliferating cell nuclear antigen (PCNA; DAKO Diagnostics, Ontario, Canada); horseradish peroxidase-conjugated secondary antibodies (Amersham Life Science, Little Chalfont, U.K.); biotinylated anti-mouse/anti-rabbit immunoglobulin G (IgG) (H+L) and horseradish peroxidase-conjugated streptavidin (Vector Laboratories, Burlingame, CA, U.S.A.). KPS-A was generously provided by Professor Hee-Juhn Park, a coauthor.[23] It was dissolved in dimethyl sulfoxide (DMSO), followed by dilution with culture medium before use. DMEM/F12, FBS, antibiotic-antimycotic solution (10000 units/mL penicillin G sodium, 10000 μg/mL streptomycin sulfate, and 25 μg/mL amphotericin B), phosphate-buffered saline (PBS), 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA), and serum- and antibiotic-free Opti-MEM were purchased from Gibco BRL (Rockville, MD, U.S.A.). 3-(4,5-Dimethylthiazol-2-yl)2,4-diphenyl tetrazolium bromide (MTT), DMSO, cholera toxin, hydrocortisone, insulin, transferrin, T3, and bovine serum albumin (BSA) were obtained from Sigma-Aldrich Chemical (St. Louis, MO, U.S.A.). PD98059 (a MEK inhibitor), SB203580 (a p38 MAPK inhibitor), SP600125 (a JNK inhibitor), LY294002 (a PI3K inhibitor), PMA, and actinomycin D were purchased from Calbiochem (La Jolla, CA, U.S.A.).

HuR or Rab1A Gene Silencing Short hairpin RNA (shRNA) or small interfering RNA (siRNA)-mediated inhibition of HuR or Rab1A was carried out according to the manufacturer's protocols. To establish HuR-knockdown YD-10B cells, the cells (3×10^4 cells/well) were transfected with 1×10^7 control or HuR shRNA (h) lentiviral particles (Santa Cruz Biotechnology) in culture medium, followed by puromycin (10 μg/mL) treatment to select the transduced cells. For Rab1A gene silencing, 100 nM human Rab1A siRNA or control siRNA (Santa Cruz Biotechnology) were introduced into YD-10B cells (3×10^4 cells/well) in serum-free Opti-MEM containing Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, U.S.A.). HuR or Rab1A protein expression was checked by Western blotting.

Cell Viability Assay YD-10B cells (5×10^3 cells/well) were plated into a 96-well culture plate with complete medium and left overnight to adhere. The cells were treated with various concentrations of KPS-A in the serum-free medium in the absence or presence of PMA (0.5 μM) for 24 h or 48 h. The cells were then incubated with a MTT solution (5 mg/mL) for an additional 4 h at 37°C. The medium was carefully removed, and the formazan product was dissolved with 200 μL diemthyl sulfoxide. Absorbance was measured at 570 nm in a microplate reader (Bio-Rad, Hercules, CA, U.S.A.).

Invasion Assay The invasive activity of the YD-10B cells was estimated using [^{3}H]thymidine-labeled cells as previously described.[23] The upper surfaces of 8 μm pore size polycarbonate nucleopore filter inserts in a 24-well Transwell chamber (Corning Costar, Cambridge, MA, U.S.A.) were coated with Matrigel (30 μg/well; Becton Dickinson, Lincoln Park, NJ, U.S.A.). The [^{3}H]thymidine-labeled cells (5×10^4 cells) with or without siRNA-mediated Rab1A inhibition were seeded into the upper part of the Matrigel-coated filter, and serum-free medium containing PMA (0.5 μM) was added to the lower part, with or without KPS-A (0—6 μg/mL), anti-MMP-9 antibody (1 μg/mL), PD98059 (50 μM), SB203580 (20 μM), SP600125 (40 μM) or LY294002 (50 μM), for 48 h. The radioactivity of the cells that invaded through the Matrigel into the lower part of the chamber was counted using a LS6500 liquid scintillation counter with a liquid scintillation cocktail (Beckman Coulter, Fullerton, CA, U.S.A.). Results are expressed as changes in invasion relative to controls.

Gelatin Zymography YD-10B cells (1×10^5 cells) were incubated in serum-free medium with KPS-A at the indicated concentrations for 2 h or with specific inhibitors of MAPKs and PI3K for 1 h, followed by stimulation with PMA (0.5 μM) for an additional 24 h. YD-10B cells with or without HuR or Rab1A knockdown were stimulated with PMA for 16 or 24 h. The conditioned medium was collected and/or the total cell lysates were prepared as described in the Western blotting section. Protein concentrations were determined by the Bradford method (Bio-Rad). The gelatinolytic activity of MMP-9 was measured as previously described.[23]

Western Blotting YD-10B cells were treated with KPS-A for 2 h or MAPK and Akt inhibitors for 1 h, and then stimulated with PMA (0.5 μM) for 16 h (Rab1A), 24 h (MMP-9, TIMP-1 and Rab1A), 6 h (HuR), or 20 min (MAPKs and Akt). The total lysates were prepared to examine the expression and/or activation of MMP-9, TIMP-1, HuR, Rab1A, MAPKs, and Akt. Cytoplasmic and nuclear fractions were obtained to examine the translocation of HuR. Equal amounts of protein (50 μg) were separated on 10% SDS-polyacrylamide gels. The proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, U.S.A.). The membrane was blocked with 5% skim milk in PBS containing 0.1% Tween-20 (PBST) and then incubated with a 1 : 1000 dilution of each primary antibody in 5% skim milk overnight at 4°C. The blots were then incubated with a 1 : 2000 dilution of the respective horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature and then washed with PBST. The targeted proteins were visualized with an enhanced chemiluminescence detection kit (Amersham Life Science) according to the manufacturer's protocol.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) To determine the effect of KPS-A on MMP-9 mRNA levels, YD-10B cells were treated with KPS-A at the indicated concentrations for 2 h and subsequently stimulated with PMA (0.5 μM) for 24 h. To study the effect

of KPS-A on MMP-9 mRNA stability, cells at 70—80% confluence in complete medium were incubated with or without KPS-A for 2 h and then stimulated with PMA for 6 h. The cells were collected at the indicated time points after actinomycin D (1 μg/mL) treatment to inhibit nascent RNA synthesis. Total RNA was isolated using the TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, U.S.A.). First-strand cDNA synthesis was performed using 2 μg of the total RNA and Promega's reverse transcription system. The following primers were used for RT-PCR: F-MMP-9, 5′-CCATTTCGACGATGACGAGTTG-3′ and R-MMP-9, 5′-CTTGTCGCTGTCAAAGTTCGAG-3′; F-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CCGCCTACTGCCCACTGCCACCAC-3′ and R-GAPDH, 5′-TCCATCCACTATGTCAGCAGGTCC-3′. PCR amplification was carried out in a reaction mixture containing 0.5 mg first-strand cDNA and 10 pmol primers and consisted of 30 cycles with the following program for each cycle: denaturation at 95°C for 30 s, annealing at 50°C (for MMP-9) or 52°C (for GAPDH) for 30 s, extension at 72°C for 1 min, and a final elongation at 72°C for 5 min. The amplified PCR product was electrophoresed on a 2% agarose gel in 1×Tris-Borate-EDTA buffer containing ethidium bromide and visualized using Quantity One software and the Gel Doc 2000 system (Bio-Rad Laboratories, CA, U.S.A.).

Quantitative RT-PCR for mRNA Expression Total RNA was isolated using the TRIzol reagent and RNA concentration was measured spectrophotometrically at an optical density of 260 nm. Quantitative reverse transcription-PCR (qRT-PCR) was performed using the GeneAmp RNA PCR Kit (Applied Biosystems, Foster City, CA, U.S.A.). Briefly, 2 μg of purified RNA per sample with Moloney murine leukemia virus reverse transcriptase and random hexamers (Promega Corporation, Madison, WI, U.S.A.). Real-time quantitative PCR analysis was performed on an ABI 7500 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, U.S.A.) with 2 μL of cDNA in a total reaction volume of 20 μL using the SYBR Green PCR mix (Eurogentec, Seraing, Belgium). GAPDH was used as a housekeeping gene for normalization. The sequences of primers in this section are the followings: (1) F-MMP-9: 5′-TGGGCTACGTGACCTATGACAT-3′, R-MMP-9: 5′-GCCCAGCCCACCTCCACTCCTC-3′; (2) F-GAPDH: 5′-TGCACCACCAACTGCTTAGC-3′, R-GAPDH: 5′-GCATGGACTGTGGTCATGAG-3′. The specificity of the products was confirmed by the melt curve analysis. No primer dimer was obtained for either MMP-9 or GAPDH as assessed by the melt curve analysis. The PCR cycles were started with Taq activation at 94°C for 5 min and followed by final extension of 72°C for 10 min. The quantitation of MMP-9 gene was normalized to amplification of GAPDH and subsequently expressed as relative to untreated control.

Luciferase Reporter Assay To measure nuclear factor-kappa B (NF-κB)- and AP-1-dependent transcriptional activities in YD-10B cells, a luciferase reporter assay was performed as described previously.[23] Cells (1×10^5 cells/well) were seeded into 6-well plates. Upon reaching 70—80% confluence, the cells were cotransfected with 0.5 μg pSV-β-galactosidase and 1 μg of pTAL-Luc vector, pNF-κB-Luc, or pAP-1-Luc reporter plasmid (Clontech, Palo Alto, CA, U.S.A.) for 8 h in serum- and antibiotics-free Opti-MEM with Lipofectamine 2000 reagent. The cells were incubated in complete medium for a further 48 h at 37°C. The transfected cells were treated with KPS-A at the indicated concentrations for 2 h and then stimulated with PMA (0.5 μM) for an additional 8 h. Luciferase and β-galactosidase activities were assayed according to the manufacturer's protocol (Promega, Madison, WI, U.S.A.) using a microplate spectrofluorometer (Molecular Devices, Palo Alto, CA, U.S.A.). Luciferase activity was normalized by the β-galactosidase activity in the cell lysate and expressed as an average of three independent experiments.

Electrophoretic Mobility Shift Assay (EMSA) YD-10B cells (1×10^5 cells) were treated with 5 μg/mL KPS-A for 2 h, followed by treatment with PMA (0.5 μM) for 1 or 2 h to determine the DNA binding activity of NF-κB and AP-1. Nuclear extracts were prepared as described in the Western blotting section, and EMSA was performed using a DNA–protein binding detection kit (Promega) according to the manufacturer's protocol. Double-stranded oligonucleotides containing the NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) or AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-3′) consensus sequences were end-labeled with Klenow [γ-^{32}P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase and purified with a NICK column (Amersham Pharmacia Biotechnology, Piscataway, NJ, U.S.A.). The eluted solutions were used as probes for EMSA. Nuclear extracts (10 μg) were incubated with the binding buffer [10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, 4% (v/v) glycerol, and 1 μg/μL poly dI-dC] for 10 min at room temperature and then treated with 0.5 pmol labeled probe for 20 min. The DNA–protein complex was separated on a 6% polyacrylamide gel in 0.5×Tris-Borate-EDTA buffer at 10 V for 3 h. The gels were dried and exposed to Kodak-XAR film.

Nude Mouse Xenograft Assay All animal studies were performed in accordance with experimental protocols that were approved by the animal ethics committee of Yonsei University College of Dentistry. Female Balb/C athymic nude mice (6 weeks of age; the Central Lab Animal, Seoul, Korea) were maintained at 20—22°C on a 12 h light/dark cycle. YD-10B cells (1×10^6 cells/0.1 mL PBS) were submucosally injected into the tongues of mice under anesthesia (n=10). Two days later, the mice began a KPS-A [1 or 5 mg/kg body weight (BW) in PBS] was orally administered three times a week for 25 d. Control mice received PBS alone instead of KPS-A. The length and width of the tumors in millimeters were measured regularly with an electric digital caliper, and tumor size was calculated by the following formula: width$^2\times$length$\times$1/2. The mice were sacrificed at the end of the treatment period, and the tongues with tumors were carefully removed for immunohistochemical analysis.

Immunohistochemical Examination The collected tongue carcinomas were fixed with 4% paraformaldehyde solution for 24 h and then embedded in paraffin. Serial tissue sections (4 m thick) were prepared and mounted onto slides. The sections were handled as previously described[23] and subsequently incubated overnight at 4°C with primary antibodies against PCNA, MMP-9, TIMP-1, HuR, or Rab1A at a dilution of 1 : 100 in PBS with 1% BSA. The sections were rinsed with PBS and incubated with biotinylated anti-mouse/anti-rabbit IgG (H+L) (1 : 100 dilution in PBS with 1% BSA) at room temperature for 30 min, followed by exposure to horseradish peroxidase streptavidin (1 : 200 dilution in PBS with 1% BSA) at room temperature for 30 min. The sections were reacted

with 0.02% 3,3′-diaminobenzidine as a chromogen, counterstained with hematoxylin, dehydrated, and mounted. Protein expressions of PCNA, MMP-9, TIMP-1, HuR, and Rab1A in tongue tissues of nude mice supplemented with 1 or 5 mg KPS-A/kg BW were quantified by Image Pro Plus software (Media Cybernetics).

Statistical Analysis Statistical analysis was conducted using InStat™ statistical software (GraphPad Software, Inc., San Diego, CA, U.S.A.). The results are expressed as means±standard error (S.E.) of three independent experiments. The statistical significance of differences between the groups was analyzed *via* repeated measures of one-way analysis of variance (ANOVA) and Student's *t*-test. A p value <0.05 was considered significant.

RESULTS

The effect of KPS-A on viability and MMP-9-mediated invasion of YD-10B OSCC cells were measured. When YD-10B cells were treated with 0—10 μg/mL KPS-A in the absence or presence of PMA (0.5 μM) for 24 or 48 h, PMA increased cell viability, but KPS-A at more than 6 μg/mL reduced the viability of both the stimulated and the unstimulated cells (Fig. 1A). In addition, PMA caused a 7.3-fold increase in the invasion of YD-10B cells, but this effect was noticeably inhibited by KPS-A treatment at the non-cytotoxic doses of 4 and 6 μg/mL (Fig. 1B). To confirm the role of MMP-9 in the PMA-induced invasion of YD-10B cells, the cells were treated with a primary antibody against MMP-9 in the presence of PMA. Neutralization with a MMP-9 antibody significantly decreased PMA-induced cell invasion (Fig. 1C). We next studied the effect of KPS-A on MMP-9 secretion and expression in PMA-treated YD-10B cells. Gelatin zymography indicated that PMA stimulation elevated the secretion of MMP-9 into the culture medium, but KPS-A dose-dependently inhibited this effect (Fig. 1D). Expression of MMP-9 protein (Fig. 1E) and mRNA (Fig. 1F) was markedly induced by PMA treatment, but this effect was abrogated by KPS-A treatment as well. The expression of MMP-9 was also confirmed by real time quantitative RT-PCR. The expression of MMP-9 increased average 25-fold in the PMA-stimulated cells as compared with the untreated control cells, whereas KPS-A treatment (6 μg/mL) decreased the PMA-induced increment 2.5-fold (Fig. 1G). In addition, PMA-induced TIMP-1 expression was blocked by KPS-A treatment (Fig. 1H). These results indicate that MMP-9 plays a crucial role in the invasion of YD-10B OSCC cells and that KPS-A inhibited cell invasion by reducing MMP-9 expression and secretion as well as TIMP-1 expression in PMA-treated YD-10B cells.

To determine how KPS-A controls MMP-9 expression in YD-10B OSCC cells, we first performed a promoter assay using YD-10B cells with a luciferase reporter gene, which includes multiple NF-κB or AP-1 binding site motifs. NF-κB-dependent luciferase activity was increased 6.5-fold, and AP-1-dependent luciferase activity was increased 2.6-fold in PMA-stimulated YD-10B cells compared with unstimulated cells. However, KPS-A treatment did not significantly suppress PMA-induced NF-κB- or AP-1-dependent luciferase activity (Fig. 2A). When we examined the DNA binding activity of these transcription factors by EMSA in YD-10B cells stimulated with PMA for 1 h (for NF-κB) or 2 h (for AP-1) after a 2 h treatment with KPS-A, we observed that KPS-A did not inhibit the PMA-induced DNA binding activity of NF-κB or AP-1 (Fig. 2B). We further investigated the effect of KPS-A on MMP-9 mRNA stability. YD-10B cells were treated with actinomycin D after KPS-A treatment and/or PMA stimulation, and mRNA levels were monitored by RT-PCR. The PMA-induced MMP-9 mRNA level was considerably reduced in the presence of actinomycin D in a time-dependent manner, and the half-life of MMP-9 mRNA was shortened by KPS-A treatment (Fig. 2C). We also confirmed whether the mRNA stabilizing factor HuR could affect MMP-9. In HuR-knockdown YD-10B cells, the MMP-9 level in the culture medium (CM) definitely reduced in the absence or presence of PMA (Fig. 2D). In addition, KPS-A treatment inhibited the increased cytoplasmic HuR level and restored its nuclear level to the control level in a dose-related manner in YD-10B cells stimulated with PMA for 6 h (Fig. 2E). Our data suggest that KPS-A downregulated MMP-9 expression in PMA-treated YD-10B OSCC cells by reducing its mRNA stability *via* the blockade of PMA-induced cytoplasmic translocation of HuR, not NF-κB- or AP-1-dependent transcriptional activation.

We determined whether KPS-A could inhibit MMP-9 secretion from YD-10B cells to the extracellular space. Gelatin zymographic analysis indicated that KPS-A treatment resulted in the intracellular accumulation of PMA-induced MMP-9 and thereby inhibited MMP-9 level in the conditioned medium (CM) of YD-10B cells (Fig. 3A). We also examined the effect of KPS-A on the expression of Rab1A, one of the small Rab GTPases that regulate the release of MMPs. PMA stimulated Rab1A expression in YD-10B cells for up to 24 h (Fig. 3B). In the PMA-treated YD-10B cells, siRNA-mediated Rab1A depletion remarkably inhibited MMP-9 level in the conditioned medium (CM) (Fig. 3C) as well as cell invasion (Fig. 3D). KPS-A treatment substantially blocked PMA-induced Rab1A expression (Fig. 3E). These results demonstrate that KPS-A reduced the PMA-induced MMP-9 secretion of YD-10B cells by inhibiting PMA-induced Rab1A expression, consequently leading to an inhibition of cell invasion.

To determine whether MAPKs or PI3K/Akt are involved in the MMP-9-mediated invasiveness of PMA-treated YD-10B cells, the cells were treated with specific inhibitors of ERK1/2, p38 MAPK, JNK, and PI3K/Akt and then stimulated by PMA. PMA-stimulated cell invasion (Fig. 4A), MMP-9 secretion (Fig. 4B), and the protein levels of MMP-9, TIMP-1, HuR, and Rab1A (Fig. 4C) were substantially inhibited by the ERK1/2 and PI3K inhibitors but not by the p38 MAPK or JNK inhibitors. KPS-A inhibited the phosphorylation of ERK1/2 and Akt in PMA-treated YD-10B cells (Fig. 4D). These results indicate that KPS-A reduced MMP-9-mediated invasion of PMA-stimulated YD-10B cells by controlling HuR and Rab1A expression *via* PI3K/Akt and ERK activation.

We tested the effects of KPS-A on tumor growth in nude mice with orthotopic tongue tumors from YD-10B cells. Tumor volume was remarkably increased after 25 d in mice inoculated with YD-10B cells, but oral administration of KPS-A caused a significant inhibition in tumor volume in a dose-related manner. Reduced body weight was observed in cancer cell-inoculated mice on days 20 and 25, but this effect was not recorded in mice that received KPS-A treatment (Figs. 5A, B). From the H&E staining results, the tumor inoculated with YD-10B cells showed well-differentiated and

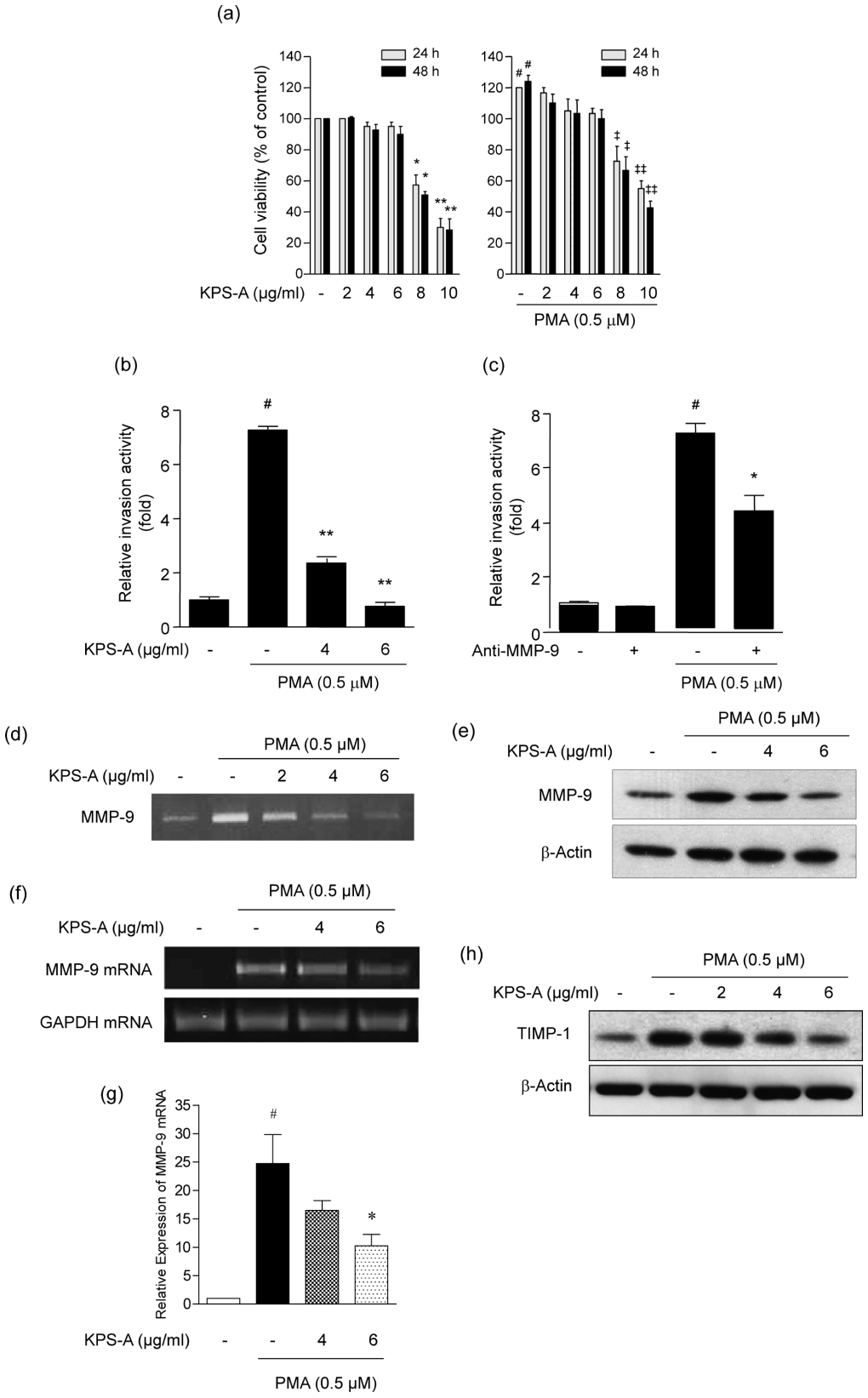


Fig. 1. KPS-A Suppresses Cell Viability and MMP-9-Mediated Invasion in PMA-Treated YD-10B OSCC Cells

(A) YD-10B cells were treated with 0—10 μg/mL KPS-A in the absence or presence of PMA (0.5 μM) for 24 or 48 h, and cell viability was determined by the MTT assay. *$p<0.05$, **$p<0.01$, $^{\#}p<0.05$ *versus* vehicle-treated cells, $^{\ddagger}p<0.05$, $^{\ddagger\ddagger}p<0.01$ *versus* PMA alone-treated cells. (B,C) [^{3}H]Thymidine-labeled YD-10B cells were treated with PMA (0.5 μM), KPS-A (4, 6 μg/mL), and/or anti-MMP-9 polyclonal antibody (1 μg/mL) for 48 h. Invasion activities were determined by a Matrigel-coated Transwell invasion assay and expressed as changes in invasion relative to controls. $^{\#}p<0.001$ *versus* vehicle-treated cells, *$p<0.05$, **$p<0.001$ *versus* PMA alone-treated cells. All data represent the mean±S.E. of three independent experiments. (D—G) Cells were treated with various concentrations of KPS-A 2 h prior to PMA (0.5 μM) stimulation. Twenty-four hours later, MMP-9 levels in the conditioned media and the expression of MMP-9 protein and mRNA were analyzed by gelatin zymography, Western blot analysis, RT-PCR, and quantitative RT-PCR (qRT-PCR), respectively. $^{\#}p<0.0001$ *versus* untreated control cells, *$p<0.05$ *versus* PMA-stimulated cells. (H) TIMP-1 protein expression was detected by Western blot analysis in the cells treated with KPS-A and PMA.

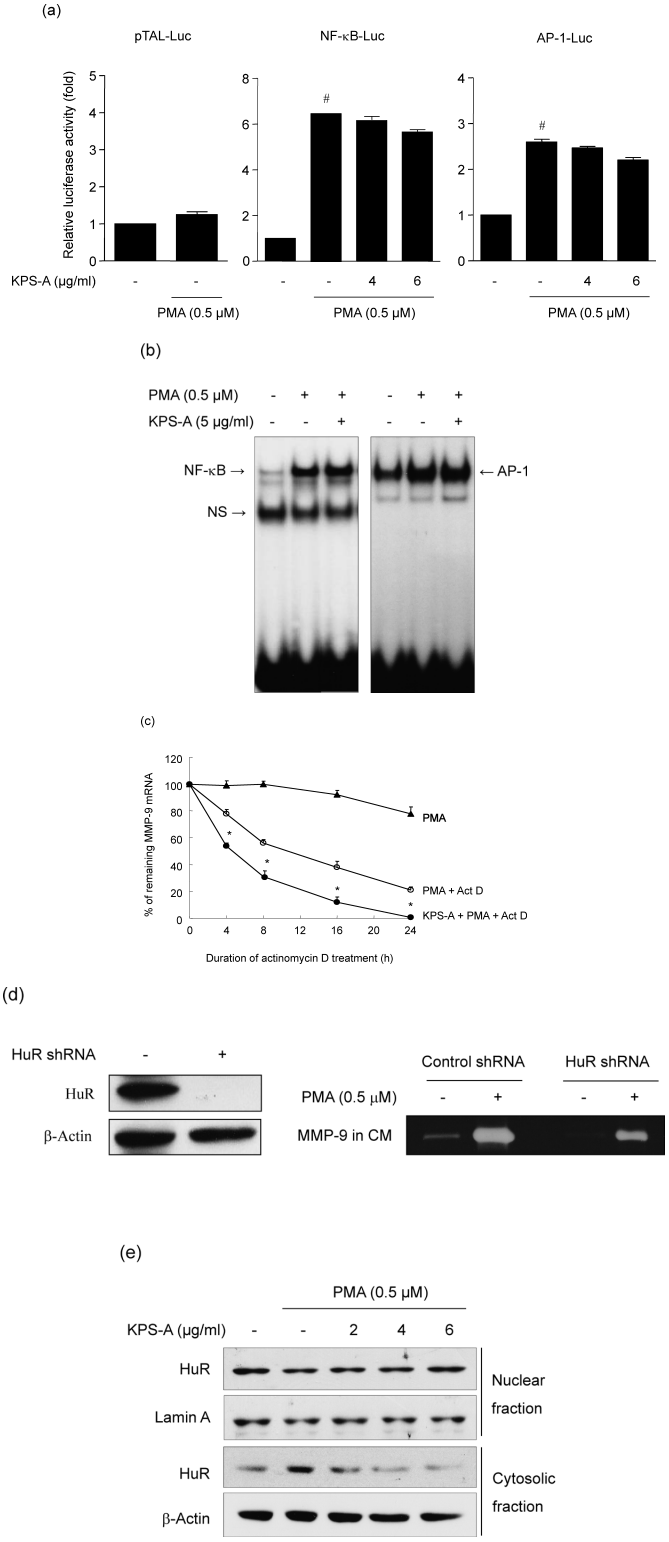


Fig. 2. KPS-A Inhibits MMP-9 Expression by Reducing mRNA Stability

(A) YD-10B OSCC cells transfected with pTAL-Luc, pNF-κB-Luc or pAP-1-Luc reporter plasmid were treated with KPS-A for 2 h and then PMA (0.5 μM) for 8 h. NF-κB- and AP-1-dependent luciferase activities were normalized to β-galactosidase activity from the cotransfection of pRSV β-galactosidase. The data represent the mean±S.E. of three independent experiments. $^{\#}p<0.001$ *versus* vehicle-treated cells. (B) YD-10B cells were treated with KPS-A (5 μg/mL) for 2 h and then stimulated with PMA for 1 h (for NF-κB) or 2 h (for AP-1). Prepared nuclear extracts were reacted with radioactive oligonucleotides containing the NF-κB or the AP-1 motif of the MMP-9 promoter. The reacted bound complexes were separated by 6% non-denaturating polyacrylamide electrophoresis. NS, non-specific band. (C) Cells were treated with KPS-A (5 μg/mL) for 2 h and then stimulated with PMA (0.5 μM) for an additional 6 h. At the indicated time points after actinomycin D (ActD) (1 μg/mL) treatment, total RNA was extracted using the TRIzol reagent, and RT-PCR was performed. MMP-9 mRNA levels were normalized to that of GAPDH. The data represent the mean±S.E. of three separate experiments. $^{*}p<0.05$ *versus* cells treated with PMA and ActD. (D) The transfected YD-10B OSCC cells with HuR shRNA or control shRNA were stimulated with PMA (0.5 μM) for 24 h. HuR protein expression and MMP-9 level in the conditioned medium (CM) were analyzed in the transfected YD-10B OSCC cells with HuR shRNA or control shRNA-A by Western blotting and gelatin zymography, respectively. (E) Cells were treated with KPS-A for 2 h, followed by PMA stimulation (0.5 μM) for 6 h. The levels of HuR in the nuclear and cytosolic fractions were analyzed by Western blotting. Lamin A and β-actin were included as loading controls for each fraction.

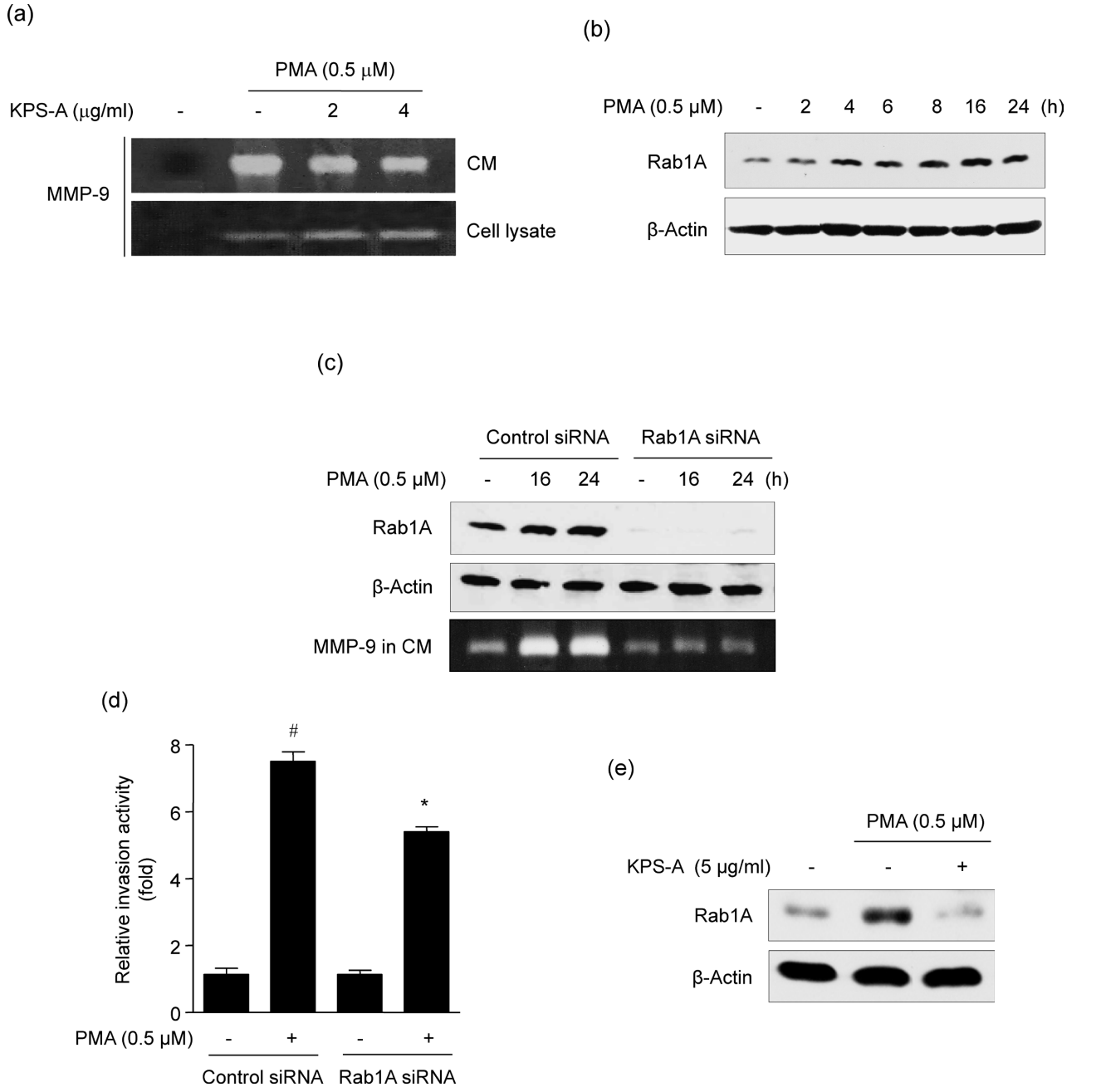


Fig. 3. KPS-A Decreases the Secretion of MMP-9 by Inhibiting Rab1A-Mediated Protein Trafficking

(A) YD-10B OSCC cells were stimulated with PMA (0.5μM) for 24 h after KPS-A treatment for 2 h. The MMP-9 level in the collected conditioned medium (CM) and in the lysate of the harvested cells was analyzed by gelatin zymography. (B) Cells were stimulated with PMA (0.5μM) for the indicated time points, and Rab1A expression was estimated by Western blotting with anti-human Rab1A antibody. (C) Cells transiently transfected with human Rab1A siRNA or control siRNA were stimulated with PMA (0.5μM) for 16 or 24 h. Rab1A expression and MMP-9 level were evaluated by Western blotting with whole cell lysate and gelatin zymography with the conditioned medium (CM), respectively. (D) The cells transfected with Rab1A siRNA or control siRNA were labeled with [^{3}H]thymidine and then allowed to invade through a Matrigel-coated Transwell chamber in the absence or presence of PMA (0.5μM) for 48 h. Invasion activity was determined by the radioactivity of the invaded cells and expressed as changes in invasion relative to the control conditions. The data represent the mean±S.E. of three independent experiments. $^{\#}p<0.001$ *versus* control siRNA-transfected cells, $^{*}p<0.05$ *versus* PMA-stimulated control siRNA-transfected cells. (E) Cells were treated with KPS-A (5μg/mL) for 2 h and then stimulated with PMA (0.5μM) for 24 h. Western blot analysis was performed with Rab1A specific antibodies. β-actin was included as a loading control in all Western blotting analyses.

highly invasive (black arrow head) SCC (T), while those of mice supplemented with 1 or 5 mg KPS-A/kg BW showed encapsulated (Fig. 5C). Immunohistochemical analysis indicated that PCNA, MMP-9, TIMP-1, HuR and Rab1A proteins were highly expressed in the tumor tissues of cancer cell-inoculated mice but not those of mice with KPS-A treatment (Fig. 5D). These results indicate that KPS-A suppressed the growth of human OSCC and cancer cell invasion-related protein expression in mice. Taken together, KPS-A may exert anti-invasive effects in OSCC.

DISCUSSION

MMPs drive the turnover of ECM molecules and interfere with cell-cell interactions and signaling molecules by cleaving and disintegrating pericellular substrates. In normal

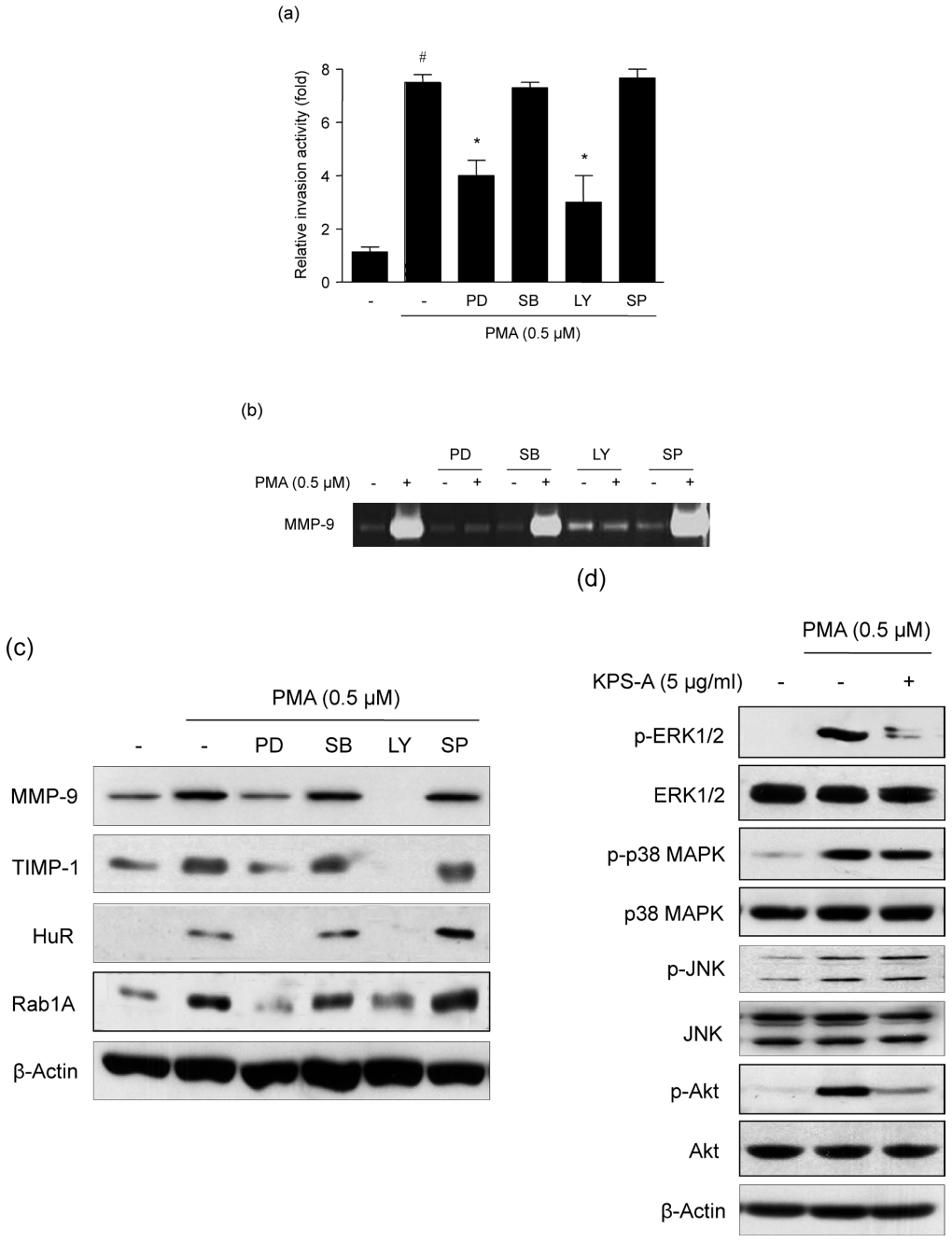
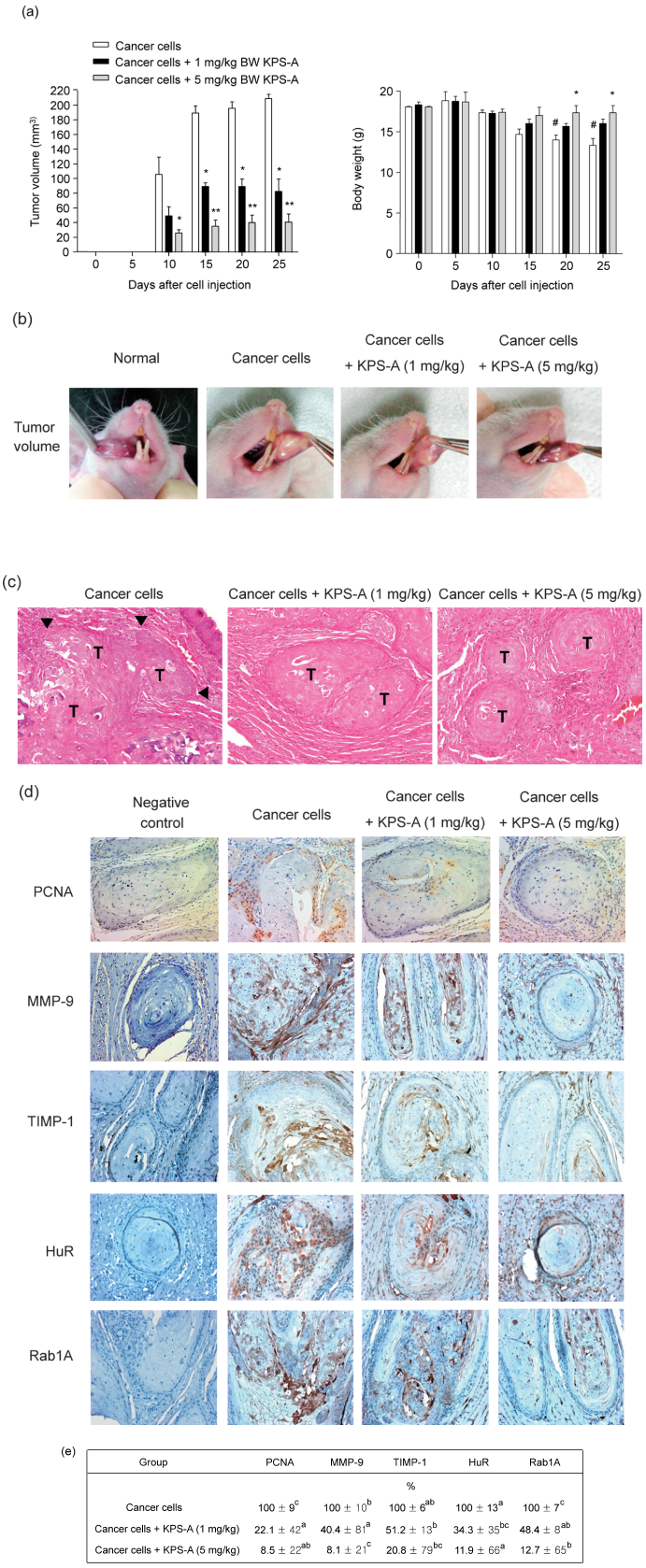


Fig. 4. PMA Stimulates HuR and Rab1A Expression *via* ERK1/2 and PI3K/Akt and KPS-A Inhibits ERK1/2 and Akt Activation in PMA-Stimulated YD-10B OSCC Cells

(A) [^{3}H]Thymidine-labeled cells were exposed to PMA (0.5 μM) in the presence or absence of specific inhibitors of MAPKs and PI3K/Akt, PD98059 (50 μM), SB203580 (20 μM), SP600125 (40 μM) and LY294002 (50 μM), for 48 h in Matrigel-coated Transwell chambers. Invasion activities were determined by the radioactivity of the invaded cells and expressed as changes in invasion relative to control conditions. The data represent the mean±S.E. of three independent experiments. $^{\#}p<0.001$ *versus* vehicle-treated cells, $^{*}p<0.001$ *versus* PMA alone-treated cells. (B, C) Cells were treated with specific inhibitors of MAPKs or PI3K/Akt for 1 h, followed by PMA (0.5 μM) for 6 h (HuR) or 24 h. (B) The conditioned media (CM) were analyzed for the secreted MMP-9 level by gelatin zymography. (C) The expression of MMP-9, TIMP-1, HuR, and Rab1A was measured in whole cell lysates by Western blotting. β-Actin was included as a loading control. (D) Cells were treated with KPS-A 2 h prior to PMA (0.5 μM) stimulation. Twenty minutes later, total and phospho-MAPKs and Akt levels were analyzed by Western blotting.

conditions, MMP activation is tightly regulated at the transcriptional level, at the posttranslational level, and by TIMPs. However, in cancer cells, excessive extracellular MMP activity induces the remodeling of the basement membrane and thereby influences the early stages of tumor initiation, growth, invasion, metastasis, and angiogenesis.[27)]

To estimate the anti-invasive activity of KPS-A, we investigated its inhibitory effects on MMP-9-mediated invasion of PMA-stimulated human OSCC cells and a murine xenograft model of human OSCC cells. We found that KPS-A, an active compound isolated from *K. pictus*, inhibited PMA-induced proliferation and invasion of YD-10B OSCC cells. Furthermore, our data showed that MMP-9 plays a critical role in the PMA-induced invasion of YD-10B cells. KPS-A reduced MMP-9 level in conditioned medium and the expression of MMP-9 protein and mRNA in PMA-treated YD-10B cells. These results indicate that KPS-A inhibits PMA-stimulated invasion of YD-10B OSCC cells by reducing MMP-9-mediated



(e)

Group	PCNA	MMP-9	TIMP-1	HuR	Rab1A
			%		
Cancer cells	100 ± 9^{c}	100 ± 10^{b}	100 ± 6^{ab}	100 ± 13^{a}	100 ± 7^{c}
Cancer cells + KPS-A (1 mg/kg)	22.1 ± 42^{a}	40.4 ± 81^{a}	51.2 ± 13^{b}	34.3 ± 35^{bc}	48.4 ± 8^{ab}
Cancer cells + KPS-A (5 mg/kg)	8.5 ± 22^{ab}	8.1 ± 21^{c}	20.8 ± 79^{bc}	11.9 ± 66^{a}	12.7 ± 65^{b}

Fig. 5. KPS-A Inhibits the Growth and Invasiveness of YD-10B OSCC in Athymic Nude Mice

KPS-A (1, 5 mg/kg BW) was administered by oral gavage to nude mice (n=10/group) orthotopically inoculated with YD-10B OSCC cells. (A) The tumor volume was calculated, and the body weight was monitored. $^{\#}p<0.05$ *versus* cancer cell-inoculated mice on day 0, $^{*}p<0.05$, $^{**}p<0.01$ *versus* cancer cell-inoculated mice. (B) Photographs representative of the tongues of inoculated nude mice with or without KPS-A administration. (C) H&E staining analysis in the tumor inoculated with YD-10B cells and those of mice supplemented with 1 or 5 mg KPS-A/kg BW. Tumor (T), invasion site (black arrow head). (D) An immunohistochemical analysis for PCNA, MMP-9, TIMP-1, HuR and Rab1A was performed on tongue tumor tissue sections using specific antibodies (×200 original magnification). (E) Quantitative assessment of immunohistochemical analysis for protein expression of PCNA, MMP-9, TIMP-1, HuR, and Rab1A in tongue tissues of nude mice supplemented with 1 or 5 mg KPS-A/kg BW. Values means±S.D., n=5. Means in a column with superscripts without a common letter differ, $p<0.05$.

ECM degradation at non-cytotoxic doses. KPS-A also inhibited PMA-induced TIMP-1 expression. TIMPs have frequently been suggested to be secreted with MMP-9 and to inhibit the proteolytic activity of MMPs.[28] However, recent studies demonstrated that high expression of both MMP-9 and TIMP-1 was present in the tumor tissues of OSCC patients with or without lymph node metastases.[2,29]

Next, we explored the molecular mechanism by which KPS-A reduces MMP-9 expression in PMA-stimulated YD-10B cells. The regulation of PMA-induced MMP-9 expression in various cancer cells has been closely associated with the NF-κB and AP-1-dependent transcriptional activation controlled by PKC, MAPKs, and PI3K/Akt.[21—23] KPS-A inhibited the transcriptional activity of NF-κB and AP-1 in MCF-7 human breast cancer cells.[23] In the present study using YD-10B OSCC cells, interestingly, KPS-A did not result in a significant inhibition of PMA-induced transcriptional activity or DNA binding of NF-κB and AP-1, although KPS-A reduced PMA-induced mRNA level of MMP-9. To distinguish the mechanism by which MMP-9 mRNA and protein expression was reduced by KPS-A treatment in YD-10B cells, we assessed MMP-9 mRNA stability by monitoring the rate of MMP-9 mRNA decay, and the stability of MMP-9 mRNA was reduced by KPS-A treatment in PMA-treated YD-10B cells. The cytoplasmic mRNA stability of many inducible genes is controlled by trans-acting factors binding to AU-rich elements (ARE) in 3′ untranslated regions (UTRs).[30,31] In searching for potential proteins that regulate mRNA turnover, we focused on the RNA-binding protein HuR, which can specifically interact with AREs within the 3′UTR and protect MMP-9 transcripts against rapid degradation.[32] While no HuR mutations have been detected in cancer, a link between HuR and malignant transformation has been suggested in cancers of the breast, colon, and ovary.[33—35] HuR is predominantly present in the nucleus of unstimulated cells, but can be exported to the cytoplasm in response to a variety of agents or proliferative and stressful signals. In the cytoplasm, HuR renders target mRNAs stable and induces their translation.[36] More recent studies showed that HuR knockdown attenuates the oncogenic potential of oral cancer cells,[31] and cytoplasmic HuR expression was closely associated with cyclooxygenase-2 expression, lymph node metastasis and distant metastasis of oral cancer cells. Thus, cytoplasmic HuR expression can be useful as an independent prognostic parameter for the reduced overall survival of oral cancer patients.[25] Our data showed that HuR knockdown substantially lowered MMP-9 level in YD-10B cells with or without PMA stimulation, and KPS-A suppressed the cytoplasmic level of HuR peaked at 6 h after PMA exposure. These results suggest that KPS-A may reduce MMP-9 expression by blocking its HuR-mediated mRNA stabilization at the post-transcriptional level.

We also attempted to determine whether KPS-A could influence MMP-9 trafficking, as this was another possible mechanism to explain the reduction in MMP-9 secretion caused by KPS-A treatment of PMA-stimulated YD-10B cells. Most MMPs are stored as inactive zymogens in secretory vesicles and are activated after secretion into the extracellular space in a Golgi-dependent pathway.[37] Exocytic trafficking of secretory vesicles is controlled by small Rab GTPases, which are regulators of membrane traffic and are common to all eukaryotic cells.[38,39] More than 60 human Rab proteins have been identified; this high number of different Rab proteins reflects their importance in the regulation of vesicle trafficking processes, including vesicle formation, motility, tethering and fusion to the acceptor membrane.[40] Defective Rab or associated proteins have been implicated in rare monogenic inherited diseases as well as common multifactorial human conditions.[41] Recent studies reported that membrane type-1 MMP proinvasive activity is regulated by a Rab8-dependent exocytic pathway in breast adenocarcinoma cells[42] and that Rab27a is a key component of the secretory machinery that regulates exocytosis in prostate carcinoma cells.[43] In addition, Rab1A-overexpression was frequently observed in tongue squamous cell carcinomas and in premalignant lesions. Thus, Rab1A can be a potential biomarker of tongue carcinogenesis.[24] These accumulating evidence indicates that vesicle trafficking and Rab proteins may be targets for tumor invasion.[44—46] To estimate the inhibitory effect of KPS-A on MMP-9 secretion, we investigated the levels of MMP-9 in cells and in the conditioned medium of PMA-treated YD-10B OSCC cells with or without KPS-A treatment. KPS-A treatment resulted in the intracellular accumulation of MMP-9, consequently suppressing PMA-induced MMP-9 level in the conditioned medium. Further experiments showed that Rab1A expression was increased by PMA stimulation. Both PMA-induced MMP-9 level in the media and cell invasion were remarkably inhibited when Rab1A was silenced by siRNA treatment. KPS-A inhibited PMA-induced Rab1A expression to nearly the control level. These results suggest that KPS-A inhibited PMA-induced MMP-9 secretion by blocking Rab1A expression. Taken together, the reduction in MMP-9 level caused by KPS-A treatment in YD-10B OSCC cells may be attributed to the decreases in both HuR-mediated mRNA stabilization and Rab1A-triggered protein secretion.

MAPKs and/or PI3K/Akt signaling molecules play important roles in oral cancer invasion as in other cancers.[47—49] To determine the upstream signaling molecules regulating MMP-9 expression in the highly invasive YD-10B OSCC cells, we assessed the effects of specific inhibitors of three MAPKs and PI3K on PMA-induced invasion and expression of related proteins. Treatment with PD98059 (an ERK inhibitor) or LY294002 (a PI3K inhibitor) suppressed PMA-induced invasion, MMP-9 level in conditioned media, and expression of MMP-9, TIMP-1, HuR and Rab1A in YD-10B OSCC cells. KPS-A treatment downregulated the PMA-induced phosphorylation of ERK1/2 and Akt. These results suggest that ERK1/2 and PI3K/Akt activation promotes MMP-9-induced invasion by increasing HuR-mediated MMP-9 mRNA stability and Rab1A-mediated MMP-9 secretion in PMA-stimulated YD-10B OSCC cells. Therefore, KPS-A treatment prevents MMP-9 expression and subsequent cell invasion by inactivating ERK1/2 and Akt.

Metastasis is a complex process that is dependent on the capacity of cancer cells to invade and migrate into adjoining cells and tissues. Therefore, cell infiltration is an essential step in the process of cancer invasion. To confirm *in vivo* the anti-invasive activity of KPS-A in oral cancer, we examined its inhibitory effect on tumor growth, invasion and MMP-9 regulatory proteins in the tongues of mice that had received orthotopic injections of YD-10B OSCC cells. Oral administration of KPS-A led to a substantial inhibition of tumor growth. Taken together, the tumor from mouse group supplemented

with 1 or 5 mg KPS-A/kg BW displayed encapsulated, caused of decreased invasion nearby stroma as compared with the KPS-A-unsupplemented cancer group which were showed well-differentiated and highly invasive. PCNA, MMP-9, TIMP-1, HuR and Rab1A expression was inhibited in the tumor tissues of mice orally administered with KPS-A, supporting our *in vitro* data that KPS-A can attenuate MMP-9-mediated invasiveness.

In conclusion, PMA stimulates the invasion of YD-10B OSCC cells by increasing HuR-dependent mRNA stability and Rab1A-mediated protein secretion of MMP-9 *via* ERK1/2 and PI3K/Akt. Conversely, KPS-A inhibits the invasiveness of oral cancer cells by blocking the PMA-induced activation of ERK1/2 and Akt signaling molecules, HuR and Rab1A expression, and finally MMP-9 expression and secretion. Therefore, we suggest KPS-A as a potent anti-invasive agents in OSCC.

Conflict of Interest

The authors state no conflict of interest.

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