

The Kazal motifs of RECK protein inhibit MMP-9 secretion and activity and reduce metastasis of lung cancer cells *in vitro* and *in vivo*

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Abstract

RECK is a membrane-anchored glycoprotein which may negatively regulate matrix metalloproteinase (MMP) activity to suppress tumor invasion and metastasis. In this study, recombinant proteins corresponding to the residues 285–368 (named as CKM which contained cysteine knot motif), 605–799 (named as K123 which contained three Kazal motifs), 676–799 (named as K23 which contained the last two Kazal motifs) and full-length RECK were produced and their anti-cancer effects were tested. Full-length RECK and K23 but not K123 and CKM inhibited MMP9 secretion and activity. In addition, RECK and K23 inhibited invasion but not migration of metastatic lung cancer cells *in vitro*. Protein binding and kinetic study indicated that K23 physically interacted with MMP-9 and inhibited its activity by a non-competitive manner. Moreover, K23 reduced metastatic tumor growth in lungs of nude mice. Taken together, our results suggest that the K23 motifs of RECK protein can inhibit MMP-9 secretion and activity and attenuate metastasis of lung cancer cells.

Keywords: RECK • kazal motif • matrix metalloproteinase • lung cancer

Introduction

Matrix metalloproteinases (MMPs) are a large family of zinc-dependent endopeptidases that participate in various cellular processes including cellular proliferation, apoptosis, tissue remodeling, angiogenesis and tumor invasion [1]. The MMP family consists of at least 23 enzymes and can be grouped into different subtypes based on the substrate specificity and sequence characteristic [2]. Control of MMPs activity can be achieved by several mechanisms. First, expression of MMPs is regulated by various growth factors, chemokines and cytokines [3]. Second, MMPs are synthesized as inactive precursors and must be activated by proteolytic cleavage [4]. The enzymes

responsive for the activation of different MMPs thus play critical roles in the regulation of MMP activity under various physiological and pathological conditions. Third, active MMPs can be negatively inhibited by natural inhibitory proteins [5]. Recently, a number of MMP inhibitory proteins including tissue inhibitor of metalloproteinases (TIMPs), thrombospondins, α 2-macroglobulin and RECK (Reversion-inducing, cysteine-rich protein with Kazal motif) have been identified to inhibit MMP activity *in vitro* and *in vivo* [6].

The RECK gene encodes a membrane-anchored glycoprotein that can negatively regulate MMP-2 and -9 activities and inhibit tumor angiogenesis and metastasis [7, 8]. This gene was isolated as a transformation suppressor gene, which induced flat reversion in v-Ki-RAS-transformed NIH/3T3 cells. While RECK mRNA is highly expressed in most of normal human tissues and untransformed cells, it is down-regulated or undetectable in many tumor cell lines or in cells that ectopically express active oncogenes [7]. Pathological investigations demonstrated that down-regulation of RECK was found in several types of human cancer including pancreatic cancer, breast cancer, non-small cell lung cancer and

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osteosarcoma [9–12]. In addition, reduced RECK expression was correlated with poor prognosis in these cancers. Our recent studies demonstrated that several oncogenes including RAS, HER-2/neu and latent membrane protein 1 (LMP-1) of Epstein-Barr virus might suppress RECK expression *via* inhibition of its transcription [13–15]. Additionally, we demonstrated that oncogenic RAS acted *via* DNA methyltransferase 3B and histone deacetylases to induce promoter methylation and deacetylation to silence RECK expression [13, 16].

Although the control of RECK gene transcription has been partially elucidated recently, the biological activities of different domains of RECK protein are largely unclear. The N-terminal of RECK protein contains five repeats of a putative cysteine knot motifs and five potential glycosylation sites [7]. In addition, a recent study demonstrated that RECK protein was glycosylated at Asn⁸⁶, Asn²⁰⁰, Asn²⁹⁷ and Asn³⁵² and glycosylation was critical for RECK to suppress tumor cell invasion by inhibiting MMP-9 secretion and repressing MMP-2 activation [17]. However, the functions of other domains are poorly understood. In this study, we investigated the anti-invasive activity of the Kazal motifs of RECK protein in cultured cells and tested their effect on the inhibition of tumor metastasis *in vivo*.

Materials and methods

Cell culture and experimental reagents

A549 and CL1.5 human lung cancer cells were obtained from the cell bank of National Health Research Institute (Maoli, Taiwan) and Dr. Kuo ML (National Taiwan University, Taiwan) respectively. Cells were routinely cultured in DMEM/F12 medium containing 10% fetal calf serum (FCS) and antibiotics. OneStep RT-PCR kit was from Qiagen (Valencia, CA, USA). Human RECK cDNA was kindly provided by Dr. Noda M. (Kyoto University, Japan). Anti-RECK antibodies were purchased from MBL (Nagoya, Japan) or BD Biosciences (San Jose, CA, USA). Anti-Hisx6 antibody was obtained from BD Biosciences and anti-actin antibody was purchased from Chemicon (Temecula, CA, USA). Antibody against MMP-9 was obtained from Oncogene Science (Cambridge, MA, USA). EnzChek MMP assay kit was purchased from Molecular Probe (Eugene, OR, USA). Bovine serum albumin (BSA) was obtained from Sigma (San Louis, MO, USA). Active recombinant MMP-9 was purchased from Calbiochem (San Diego, CA, USA).

Construction of expression vectors and purification of recombinant proteins

pSecTag2 expression vector was obtained from Invitrogen (Carlsbad, CA, USA). This vector was designed for the production of recombinant protein in mammalian cells with the proteins were fused at the N-terminus to the murine Ig κ-chain leader sequence for protein secretion and at the C-terminus to a peptide containing c-myc epitope and Hisx6 tag for protein detection and purification. pSecTag2-RECK was generated by deletion of nucleotides corresponding to the C-terminal 23 amino acids of human RECK cDNA to remove the GPI-anchored region and subcloned into the pSecTag2 vector. CKM, K123 and K23 were constructed by amplifying the cDNA corresponding to the 285–368, 605–799 and 676–799 residues of

RECK protein by PCR primers flanked with BamH I and Xho I or Hind III restriction enzyme sites. After PCR reaction, the amplified DNA fragments were subcloned into pSecTag2 vector. All of the constructed vectors were verified by DNA sequencing. Expression vectors were transfected into 293T cells and conditioned medium was collected from 48 to 96 hrs after transfection. The secreted recombinant proteins were purified by using a nickel-chelating column. The elute was further concentrated by using the 10K MWCO dialysis cassette (PIERCE) to remove the high concentration of imidazole and sterilized with a 0.22 μm filter. Purity of these recombinant proteins was checked by HPLC and was >85%. Recombinant proteins were stored at –80°C until use.

Invasion and migration assays

In vitro invasion assay was performed by using 24-well transwell units with poly-carbonate filters (pore size 8 μm) coated on the upper side with Matrigel (Becton Dickinson Labware, Bedford, MA). The lower part of the transwell unit was filled with 10% FCS medium. A total of 5 × 10³ cells in 100 μl of medium containing different recombinant proteins were placed in the upper part of the transwell unit and allowed to invade for 24 hrs. After incubation, non-invaded cells on the upper part of the membrane were removed with a cotton swab. Invaded cells on the bottom surface of the membrane were fixed in formaldehyde, stained with Giemsa solution and counted under a microscope. Migration assay was performed by similar procedures except the polycarbonate filters were not coated with Matrigel.

MMP activity assay

Conditioned medium of A549 or CL1.5 cells was harvested and incubated with various concentrations of BSA, CKM, K123, K23 or full-length RECK (as a positive control). The MMP activity was studied by the EnzChek MMP assay kit using fluorescein-conjugated gelatin (DQTM gelatin) as substrate and purified type IV collagenase as a positive control enzyme. Experiments were carried out according to the procedures of the manufacturer and the output signal (excitation wavelength, 495 nm; emission wavelength, 520 nm) was recorded for 2 min at 37°C by using a fluorescent reader (BMG LABTECH, Offenburg, Germany). Results of three independent experiments were expressed as mean ± SE.

Kinetics of K23 on MMP-9 activity

To study the kinetics of MMP-9 inhibition, active recombinant MMP-9 (5 ng) was mixed with various concentrations of a fluorescein-conjugated gelatin (DQTM gelatin, Molecular Probe) and recombinant K23 protein (0, 41.7 or 125 nM). Experiments were performed similar to the procedures of MMP assay. The output signal (excitation wavelength, 495 nm; emission wavelength, 520 nm) was recorded for 2 min at 37°C by using a fluorescent reader (BMG LABTECH) and the dissociation constant was calculated.

Gelatin zymography

Zymography assay was performed as described previously [15]. BSA or recombinant proteins were added into the culture medium of A549

cells and incubated for 24 hrs. Conditioned medium was collected and concentrated by using Centricon YM-50 columns (Amicon, Bedford, MA, USA). Cell number was determined by using the hemocytometer. Conditioned medium from an equal number of cells was separated by 10% acrylamide gels containing 0.1% gelatin (Invitrogen). The gels were incubated in 2.5% Triton X-100 solution at room temperature with gentle agitation to remove SDS and were soaked in reaction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM CaCl₂ and 0.5 mM ZnCl₂) at 37°C overnight. After reaction, the gels were stained for 1 hr with staining solution (0.1% w/v Coomassie Brilliant Blue, 30% v/v methanol and 10% v/v acetic acid) and were destained in the same solution, but without Coomassie Brilliant Blue. Gelatinolytic activity of MMPs was visualized as a clear band against a dark background of stained gelatin.

Western blot analysis and immunoprecipitation assay

Cells were harvested and equal amount of cellular proteins was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously [18]. Proteins were transferred to nitrocellulose membranes and the blots were probed with various primary antibodies. Enhanced chemiluminescence reagents were used to detect the proteins on the membranes. For study of the interaction between K23 and MMP-9, active recombinant MMP-9 protein (100 ng) was incubated with K23 (500 ng) at 4°C for 2 hrs. Anti-MMP-9 antibody was added and incubated at 4°C overnight with gentle rotation. The immunocomplex was precipitated by protein G-agarose beads and subjected to SDS-PAGE. K23 was detected by using anti-Hisx6 antibody.

RT-PCR

Cells were pre-incubated without or with K23 (3 µg/ml) for 2 hrs and then stimulated with epidermal growth factor (200 ng/ml) for another 1 hr. Total RNA was extracted and RT-PCR was performed to investigate the MMP-9 expression as described previously [18]. The primers used are: MMP-9-forward: 5'-CACTGTCCACCCCTCA GAGC-3'; MMP-9-reverse: 5'-GCCACTTGTCGGCGATAAGG-3'; GAPDH- forward: 5'-CCCATCAC-CATCTTCAG-3' and GAPDH-reverse: 5'-CAGTCTTCT GGGTGGCAGT-3'.

Establishment of K23-expressing stable transfectants and experimental metastasis assay

A549 cell were transfected with pSecTag2 (control) or K23-expressing pSecTag2 (K23) vectors by Lipofectamine2000 reagent. Stable transfectants were selected by continuous treatment of hygromycin (400 µg/ml) for 4 weeks and expression of K23 was examined by Western blot analysis. 1 × 10⁶ cells in 0.2 ml PBS were injected into the tail vein of the BALB/c nude mice. Eight weeks after inoculation, animals were sacrificed and the lungs of the animals were inspected for metastatic nodules as described in our previous study [19]. Each group contained five mice. Metastatic nodules were averaged and expressed as Mean ± SE. Animal studies were approved by the Animal Care and Ethics Committee of the National Sun Yat-Sen University.

Statistical analysis

Student's t-test was used to evaluate the difference between various experimental groups. Differences were considered to be significant at $P < 0.05$.

Results

Production of full-length and various deletion polypeptides of RECK protein

To clarify the biological activity of Kazal motifs of RECK protein, we generated various deletion constructs and tried to produce recombinant proteins by using mammalian (pSecTag2) expression systems. Because RECK is a glycosylphosphatidylinositol (GPI)-anchored protein and needs glycosylation for its activity, we deleted the C-terminal 23 amino acids and subcloned the cDNA into the pSecTag2 expression vector. The vector was transfected into 293T cells and the Hisx6-tagged full-length RECK protein secreted into the conditioned medium was purified by a nickel-chelating resin. Eluted proteins were further concentrated by using molecular weight cut-off (MWCO) dialysis cassette to remove the high concentration of imidazole and sterilized with a 0.22 µm filter. Purity of these recombinant proteins was checked by HPLC and was >85%. As shown in Fig. 1A, Western blotting indicated the transfected 293T cells produced high amount of recombinant full-length RECK protein with a molecular weight about 110 kD. Recombinant proteins corresponding to the residues 605–799 (named as K123 which contained three Kazal motifs) and 676–799 (named as K23 which contained the last two Kazal motifs) were produced similarly (Fig. 1B and C). Because the recombinant proteins contained an N-terminal murine Ig κ-chain leader sequence and a C-terminal c-myc epitope and Hisx6 tag sequence, the molecular weights of K123 and K23 were estimated to be 40 and 22 kD, respectively. Western blot analysis demonstrated that specific bands representing the recombinant proteins were detected in the predicted positions. Another recombinant protein corresponding to the residues 285–368 (named as CKM which contained cysteine knot motif) was produced. Two protein bands were appeared in the Western blot because CKM was glycosylated on Asn²⁹⁷ and Asn³⁵² (Fig. 1D). The glycosylation of CKM was confirmed by treatment of glycosidase and a reduction of molecular weight of the protein to 11 kD corresponding to the non-glycosylated form was observed (data not shown).

Kazal motifs of RECK protein inhibited cell invasion but not migration

We tested whether the recombinant proteins affected migration of two highly metastatic lung cancer cell lines A549 and CL1.5. Similar to the results reported in another highly invasive HT1080 fibrosarcoma cells, we found that full-length RECK did not inhibit

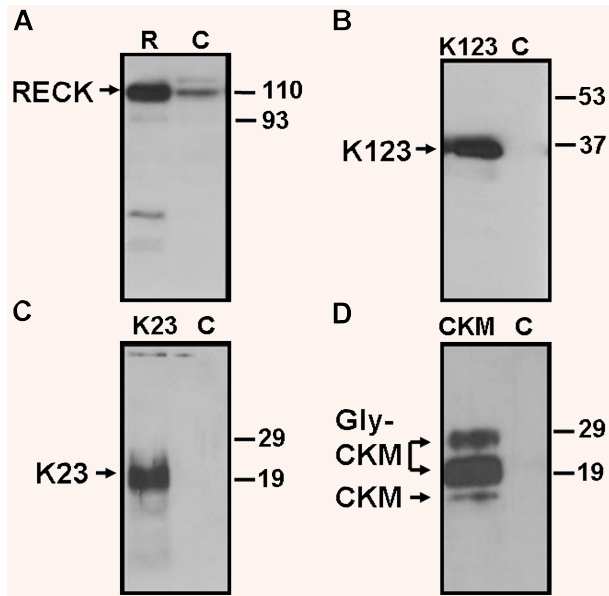


Fig. 1 Production of full-length and functional motifs of RECK protein. (A) Full-length RECK expression vector was generated by subcloning human RECK cDNA into the pSecTag2 vector and transfected into 293T cells. Conditioned medium was collected and the secreted recombinant proteins were purified by using a nickel-chelating resin. Western blot analysis was performed to detect Hisx6-tagged RECK protein. (B) K123 expression vector was constructed by amplifying the cDNA by PCR reaction and the amplified DNA fragment was subcloned into pSecTag2 vector for protein production. (C) K23 expression vector was constructed by subcloning the PCR-amplified DNA fragment into pSecTag2 vector for protein production. (D) CKM protein was produced as described above and purified by a nickel-chelating resin. The glycosylated forms of CKM protein (Gly-CKM) were also indicated.

the migration of A549 and CL1.5 lung cancer cells (Fig. 2A and B). Our data also showed that CKM, K123 and K23 did not affect the migration of A549 and CL1.5 cells. We next studied the effect of these recombinant proteins on cell invasive ability and our results demonstrated that CKM at 3 $\mu\text{g/ml}$ showed marginal inhibition of invasion of A549 and CL1.5 lung cancer cells (Fig. 2C and D). On the contrary, full-length RECK and K23 inhibited cell invasion in a dose-dependent manner (Fig. 2C and D). In addition, A549 cells were more sensitive to RECK and K23 than CL1.5 cells. Unexpectedly, K123 could not significantly repress the invasion of A549 and CL1.5 cells although a marginal reduction of invaded cell number was found when high concentration (3 $\mu\text{g/ml}$) of K123 was used.

K23 inhibited MMP-9 activity and secretion

Because K23 inhibited cell invasion but not migration, it seemed that K23 might affect the MMP activity to reduce the degradation

of Matrigel and to attenuate cell invasion. Therefore, we examined the total MMP activity in the conditioned medium by using the EnzChek MMP assay kit. As shown in Fig. 3A and B, full-length RECK or K23 significantly reduced the MMP activity of A549 and CL1.5 cells. Conversely, CKM and K123 had no effect. Since the recombinant proteins were exogenously added, these data suggested that full-length RECK and K23 directly inhibited the enzymatic activity of MMP. RECK had also been demonstrated to reduce the secretion of MMP-9 *via* an unknown mechanism [7], so we tested whether K23 might affect MMP-9 release. A549 cells were used and recombinant proteins were added directly into the cultured medium for 24 hrs. Conditioned medium was collected and subjected to gelatin zymography assay. As expected, we found that full-length RECK reduced the pro-form and active form of MMP-9 in the conditioned medium (Fig. 3C). This data was consistent with the previous work showing that RECK inhibited MMP-9 secretion [7]. Secretion of pro-MMP-2 and active MMP-2 were not affected by RECK (data not shown). Under the same experimental condition, K23 also potently attenuated the amount of pro-form and active form of MMP-9 in the conditioned medium while K123 had no effect. These results were in agreement with the data of our MMP enzymatic assay and suggested that K23 repressed MMP-9 secretion and activity simultaneously. We also tested whether K23 affected epidermal growth factor (EGF)-stimulated MMP-9 gene transcription. Our data indicated EGF up-regulated MMP-9 mRNA by 2.5–3 folds and K23 did not significantly inhibit the increase of MMP-9 mRNA level by EGF in A549 cells (Fig. 3D). However, EGF-stimulated secretion and activation of MMP-9 was significantly attenuated by K23 (Fig. 3E). Therefore, K23 is effectively in the inhibition of basal- and growth factor-stimulated MMP-9 secretion and activation.

K23 functioned as a non-competitive inhibitor for MMP-9

Because K23 directly inhibited MMP-9 activity, we predicted that K23 might physically interact with MMP-9. We incubated K23 with active MMP-9 and immuno-precipitated MMP-9 with a specific antibody. Our data clearly demonstrated that K23 bound with active MMP-9 *in vitro* (Fig. 4A). This binding is specific because immunoblotting of MMP-9 verified that equal amount of MMP-9 was immunoprecipitated by the MMP-9 antibody and non-immune Ig could not pull down the MMP-9/K23 complex (data not shown). We next investigated the mechanism by which K23 inhibited MMP-9. Kinetic analysis indicated that K23 inhibited MMP-9 activity in a non-competitive manner with the dissociation constant <15.5 nM (Fig. 4B).

Expression of K23 suppressed tumor metastasis *in vivo*

Our aforementioned results verified that K23 potently inhibited MMP-9 activity and invasion of lung cancer cells *in vitro*. We next

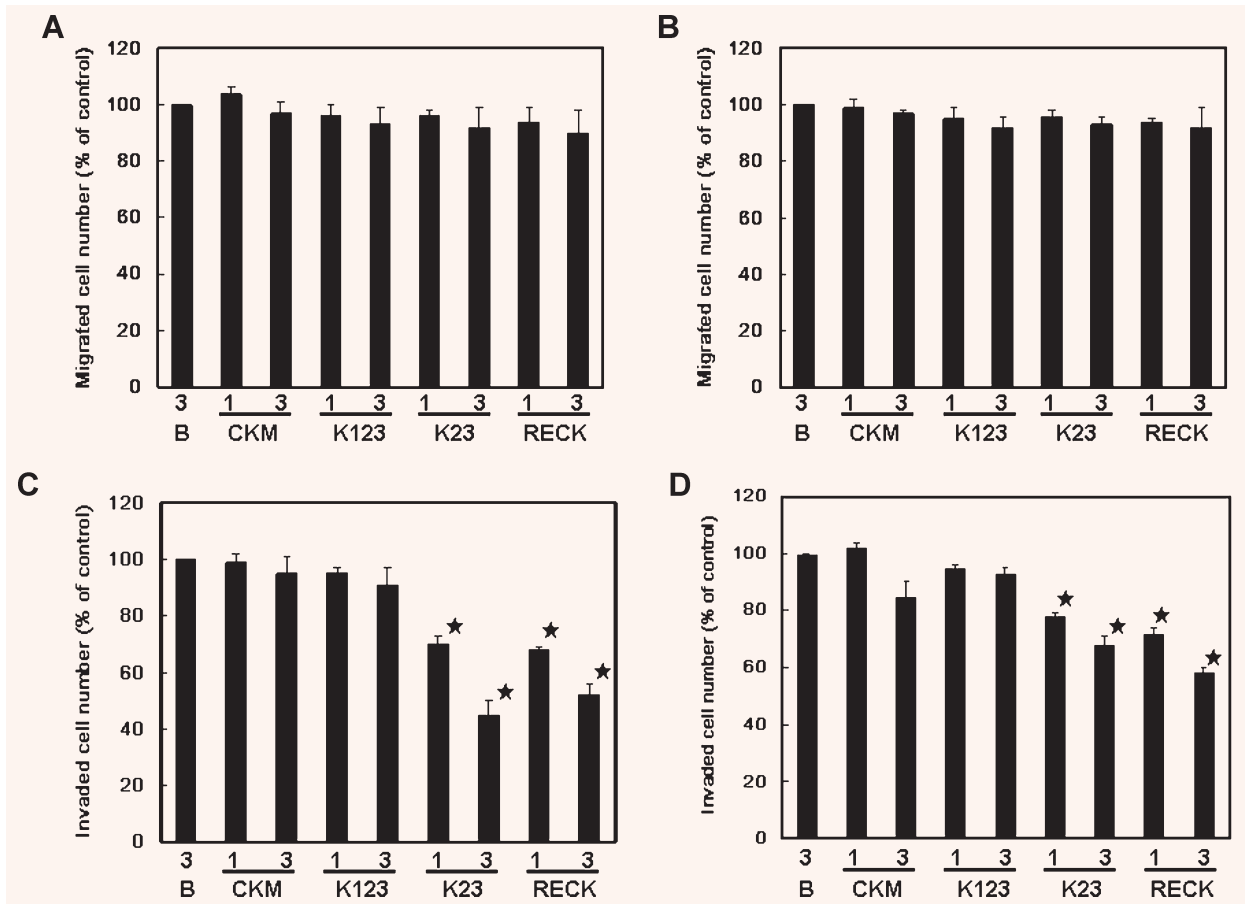


Fig. 2 Inhibition of cell invasion by full-length RECK and K23. **(A)** A549 cells were incubated with BSA **(B)** or various recombinant proteins (1 or 3 μg/ml) and placed in the upper part of the transwell unit for migration assay. The number of migrated cells of the group treated with 3 μg/ml of BSA **(B)** was defined as 100% (n = 3). **(B)** CL1.5 cells were treated as described above and the effect of various recombinant proteins on cell migration was studied (n = 3). **(C)** A549 cells in mediums containing different recombinant proteins were placed in the upper part of the transwell unit coated with Matrigel. The number of invaded cells of the control group treated with 3 μg/ml of BSA was defined as 100% (n = 3). *P < 0.05 when the results of recombinant protein-treated groups were compared with the results of control group. **(D)** CL1.5 cells were subjected to *in vitro* invasion assay as described above (n = 3). *P < 0.05 when the results of recombinant protein-treated groups were compared with the results of control group.

studied whether K23 indeed functioned as a metastasis suppressor *in vivo*. A549 cells were transfected with control (pcDNA3.1) or K23 expression vector. Stable transfectants were selected by treatment of hygromycin (400 μg/ml) for 4 weeks. As shown in Fig. 5A, K23 transfectants indeed expressed high level of K23 protein in the culture medium while no signals were found in cells transfected with control vector. Stable transfectants were pooled and used for invasion assay. We found that expression of K23 in A549 cells reduced their invasion ability by about 45–50% (Fig. 5B). Pooled stable transfectants were also injected into mice *via* tail vein. After 8 weeks, mice were sacrificed and lungs were stained by intra-trachea injection of India ink. We found that metastatic tumor nodules were identified in the lungs (Fig. 5C).

The average number of tumor nodules in mice injected with control vector-transfected A549 cells was 23 ± 7 per mice (n = 5). Conversely, the average number of nodules was reduced to 10 ± 4 per mice (n = 5, Fig. 5D). Therefore, expression of K23 protein significantly induced a 57% of reduction of metastatic tumor nodules *in vivo*.

Discussion

In this study, we demonstrate that the K23 domain of RECK protein inhibits MMP-9 secretion and activity and reduces metas-

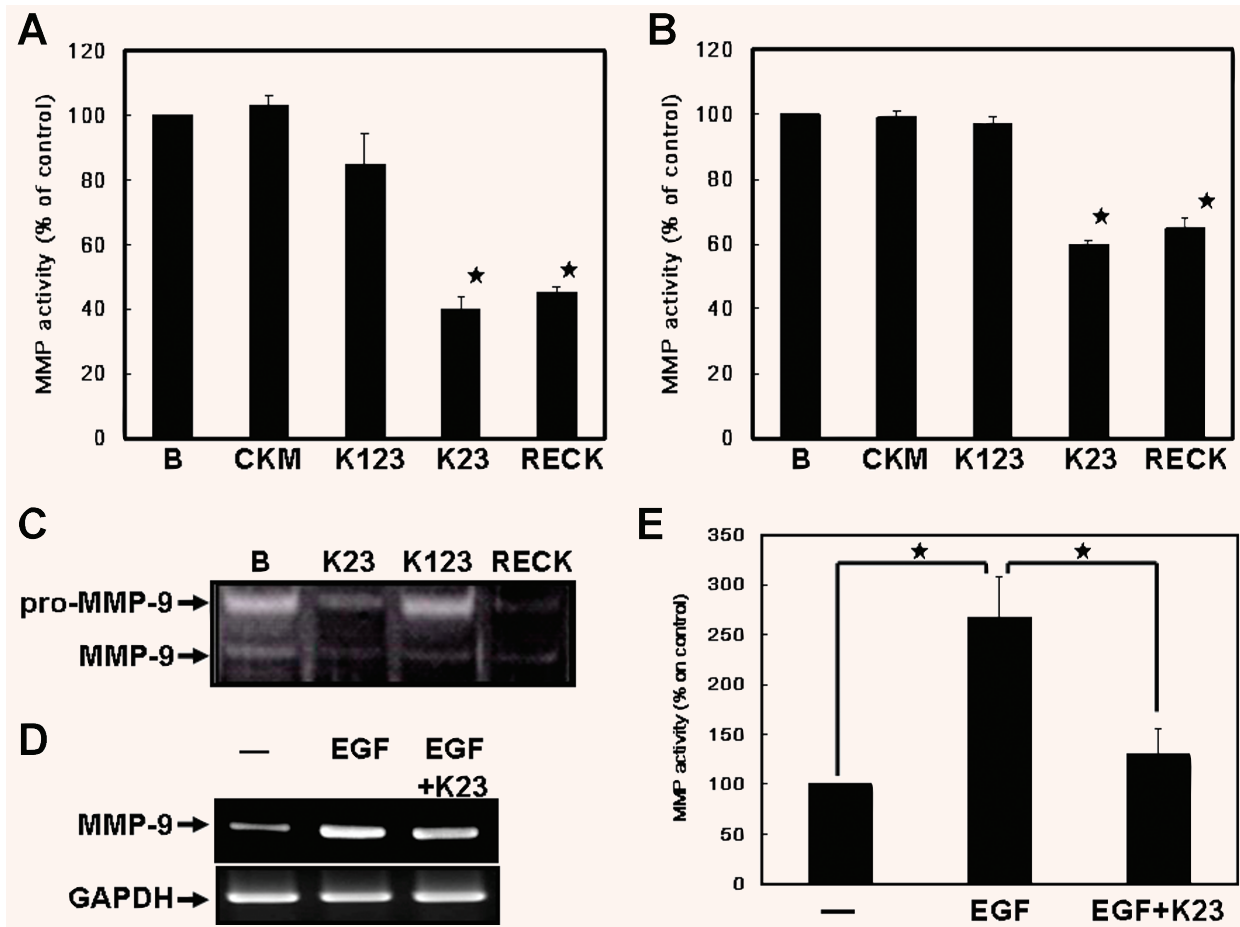


Fig. 3 Full-length RECK and K23 inhibited MMP-9 secretion and activity. **(A)** Conditioned medium of A549 cells was incubated with 3 $\mu\text{g/ml}$ of BSA **(B)**, CKM, K123, K23 or full-length RECK at 37°C for 30 min. The MMP activity was studied as described in Materials and Methods. Results of three independent experiments were expressed as mean \pm SE. MMP activity of the control group incubated with 3 $\mu\text{g/ml}$ of BSA was defined as 100% ($n = 3$). * $P < 0.05$ when the results of recombinant protein-treated groups were compared with the results of control group. **(B)** Conditioned medium of CL1.5 cells was also subjected to MMP activity assay as described above ($n = 3$). * $P < 0.05$ when the results of recombinant protein-treated groups were compared with the results of control group. **(C)** BSA and recombinant proteins (3 $\mu\text{g/ml}$) were added into the culture medium of A549 cells and incubated for 24 hrs. Conditioned medium from an equal number of cells was subjected to gelatin zymography analysis. The positions of pro-MMP-9 and active MMP-9 were indicated. **(D)** Cells were treated without (—) or with EGF (200 ng/ml) in the absence or presence of K23 (3 $\mu\text{g/ml}$) for 4 hrs and RT-PCR was performed to investigate MMP-9 mRNA level. **(E)** Cells were treated as described above and conditioned medium was collected at 24 hrs after treatment. MMP activity of the conditioned medium was studied ($n = 3$). * $P < 0.05$ when the results of two experimental groups were compared.

tasis of lung cancer cells *in vitro* and *in vivo*. Three critical issues should be discussed. First, we have identified a new peptide inhibitor for MMP-9 in this study. Among MMPs identified in mammalian cells, MMP-2 and MMP-9 are strongly associated with tumor metastasis in various human cancers [20, 21]. Therefore, MMP inhibitors either macromolecular inhibitors (TIMPs and monoclonal antibodies) or small chemical inhibitors

(synthetic and natural products) are potential agents for the therapy of diseases in which excess MMP activity (like cancer and arthritis) has been implicated. However, results from clinical trials for cancer treatment were disappointing [22]. Previous studies suggested that invasion and metastasis, which MMPs contributed heavily during tumor progression were late events in cancer development. However, recent studies clearly indicated that invasion

and metastasis could occur during early stages as well [23]. Therefore, the appropriate time for the application of MMP inhibitors should be re-evaluated. Recently, several hydroxamate-based MMP inhibitors including Marimastat, SC903, CGS27023A have been developed because hydroxamate has potent zinc-chelating activity that may block the active site of most MMPs [24–27]. However, these small inhibitors exhibit less substrate specificity and block a broad spectrum of MMPs [28]. Conversely, few peptide inhibitors have been developed until now. One peptide inhibitor Regasepin 1 with the amino acid sequence of PRCBGE has been identified recently [29]. Cell-based study shows that Regasepin 1 inhibits MMP-8 and MMP-9 with a 50% inhibitory dose (ID50) of 3 and 1 μM , respectively [30]. In this study, we demonstrate that K23 is a potent inhibitor for MMP-9. Kinetic analysis indicates that ID50 of K23 (<15.5 nM) is much lower than that of Regasepin 1 indicating K23 is a more potent MMP-9 inhibitor. In addition, K23 is a non-competitive inhibitor, which is different from most of the substrate-based inhibitors developed recently. Our animal study also verifies that K23 indeed represses the metastasis of lung cancer cells. Thus, we have identified a potential peptide inhibitor for MMP-9.

The second important issue is the potent *in vivo* efficacy and *in vivo* targets of K23 in lung metastasis. Our cell-based experiments demonstrated that only 30–40% of inhibition of cell invasion was found after treatment of K23. However, a 60% of inhibition of lung metastasis was observed in animal studies. Because MMP-9 has been verified to be a direct target for K23 in cultured cells, it is reasonable to hypothesize that inhibition of MMP-9 is one of the mechanisms for K23 to repress tumor metastasis. However, it is notable that there is a large contribution of non-cancer cells in MMP-9 expression in tumors. MMP-9 can be produced by stromal fibroblasts [20, 31]. In addition, a variety of immune cells including macrophages, monocytes, dendritic cells, natural killer cells, lymphocytes and neutrophils can express and produce MMP-9 [32]. Therefore, MMP-9 released by immune cells of the experimental animals may play some roles in the promotion of lung tumor metastasis, and K23 may inhibit all of MMP-9 produced by cancer and immune cells that causes a more profound effect in the inhibition of metastasis *in vivo*. Another possibility is there are some other *in vivo* targets of K23. Several proteases including MMP-2, MMP-3, MMP-7, MMP-10, MMP-13 and MMP-26 have been shown to be key regulators for the activation of MMP-9. So, K23 may repress the enzymatic activity of these MMPs to attenuate MMP-9 activation and to reduce metastasis. It is also possible that these MMPs are involved in the induction of lung metastasis and can be directly inhibited by K23. An important issue for further investigations is how many kinds of MMP are direct targets for K23.

The third issue is the inhibition of MMP-9 secretion by K23. Until recently, the molecular mechanism, which controls the secretion of MMP-9 is still unclear. By using human melanoma cells as a model, Schnaeker *et al.* demonstrated that MMP-9 was stored as small intracellular vesicles, which localized near the cell lamellipodia [33]. Double staining revealed that MMP-9 was

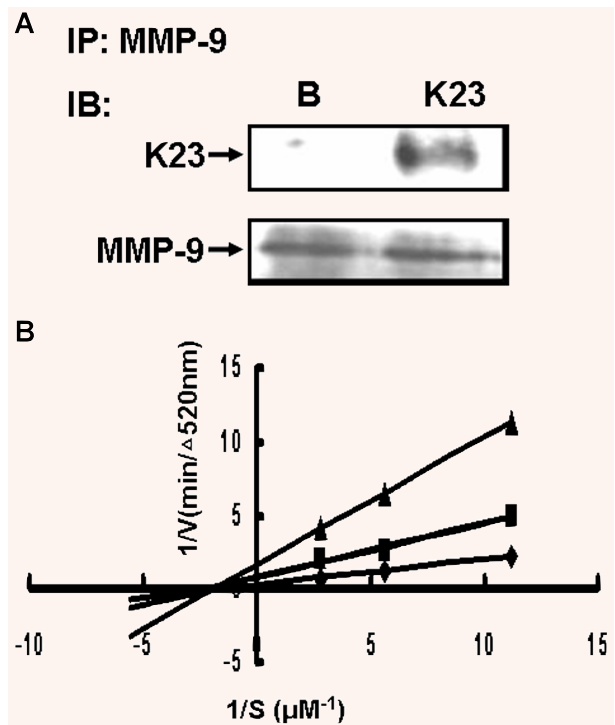


Fig. 4 K23 physically interacted with active MMP-9 and functioned as a non-competitive inhibitor. **(A)** Active recombinant MMP-9 protein (100 ng) was incubated with BSA **(B)** or K23 (500 ng) at 4°C for 2 hrs. Anti-MMP-9 antibody was added and the immunocomplex was precipitated by protein G-agarose beads. The binding of K23 to MMP-9 was detected by using anti-His6 antibody and the blots were also probed with anti-MMP-9 antibody to confirm that the equal amount of MMP-9 was immunoprecipitated. **(B)** Active recombinant MMP-9 (5 ng) was mixed with various concentrations of a fluorescein-conjugated gelatin (DQ™ gelatin) and recombinant K23 protein (0, 41.7 or 125 nM). Experiments were performed similar to the procedures of MMP assay as described in Materials and methods.

strongly associated with α -tubulin and the MMP-9-containing vesicles were distributed along the microtubular network. In addition, most of the vesicles were also co-localized with another motor protein kinesin. Some intracellular or extracellular signals may stimulate the fusion of the vesicles and plasma membrane and then trigger the secretion of MMP-9. It seems possible that K23 may interact with specific membrane proteins and block the signal-induced fusion process to inhibit MMP-9 release. Identification of these membrane proteins will lead to the understanding of the mechanism by which K23 suppresses MMP-9 secretion.

The lack of inhibitory function of K123 on cell invasion is somehow surprising because K123 contains the amino acid

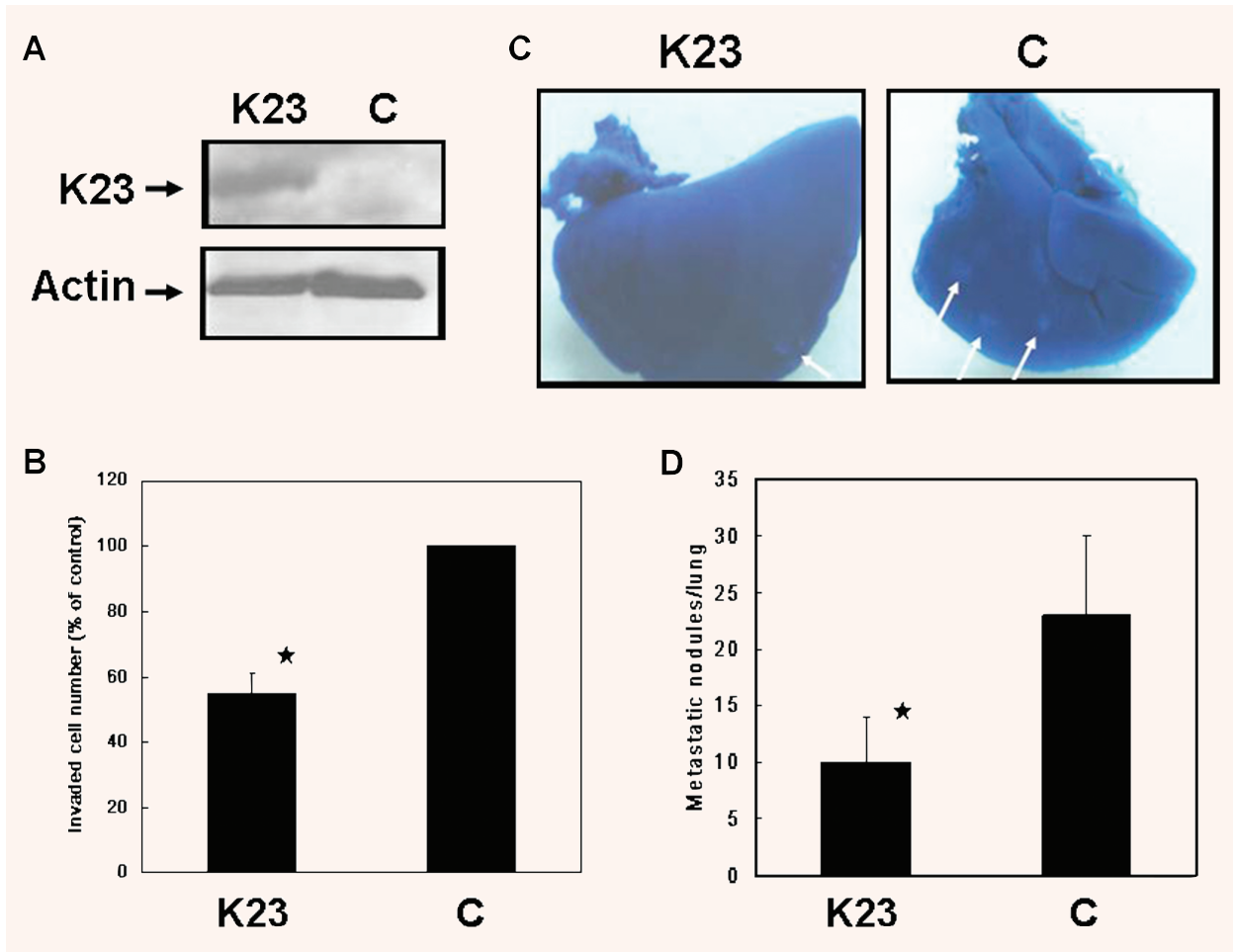


Fig. 5 Inhibition of *in vivo* metastasis of lung cancer cells by K23. (A) A549 stable transfectants expressing pSecTag2 (C) or K23-expressing pSecTag2 (K23) vectors were selected by continuous treatment of hygromycin. Expression of K23 protein was examined by Western blot analysis. (B) The invasive ability of the stable transfectants was investigated as described in Fig. 2C. The invaded cell number of cells transfected with pSecTag2 (C) was defined as 100% (n = 3). **P* < 0.05 when the results of K23-expressing group was compared with the results of control group. (C) A total of 1×10^6 stable transfectants were injected into the tail vein of the BALB/c nude mice. Eight weeks after inoculation, animals were sacrificed and the lungs of the animals were inspected for metastatic nodules (indicated by arrow sign). Typical figures of lungs from the two experimental groups were shown. (D) Metastatic nodules were averaged and expressed as Mean \pm SE (n = 5). **P* < 0.05 when the results of K23-expressing group were compared with the results of control group.

sequence completely matching to the Kazal motif at its N-terminal region. One possible explanation of this result is that K123 may exhibit some misfolding in the secondary or tertiary structure because many cysteine residues are existed in this recombinant protein, which may lead to mismatch pairing of disulfide bonds and distort its native conformation. Taken together, results of this study suggest that the Kazal motifs of RECK protein K23 is a potent inhibitor for MMP-9 secretion and activity and may inhibit the metastasis of lung cancer cells.

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