

RECK Negatively Regulates Matrix Metalloproteinase-9 Transcription

Satoshi Takagi,^{1,2} Siro Simizu,¹ and Hiroyuki Osada^{1,2}

¹Antibiotics Laboratory and Chemical Biology Department, Advanced Science Institute, RIKEN and ²Graduate School of Science and Engineering, Saitama University, Saitama, Japan

Abstract

RECK, a glycosylphosphatidylinositol-anchored glycoprotein, inhibits the enzymatic activities of some matrix metalloproteinases (MMP), thereby suppressing tumor cell metastasis; however, the detailed mechanism is still obscure. In this study, we compared the gene expression profiles between mock- and RECK-transfected HT1080 cells and showed that RECK decreases MMP-9 mRNA levels but not other MMP mRNA levels. Moreover, treatment with RECK-specific siRNA increased MMP-9 mRNA in RECK-expressing cells. The promoter assay showed that MMP-9 promoter activity was suppressed by RECK and that RECK-mediated suppression of MMP-9 promoter activity requires 12-O-tetradecanoylphorbol-13-acetate-responsive element (TRE) and κ B sites. Moreover, the binding ability of Fra-1 and c-Jun to TRE within the MMP-9 promoter region was suppressed by RECK. Thus, these results show that RECK is a negative regulator of MMP-9 transcription. [Cancer Res 2009;69(4):1502–8]

Introduction

Matrix metalloproteinases (MMP) play key roles in the degradation of extracellular matrix (ECM) that is associated with homeostasis. On the other hand, in cancer, MMPs contribute to the formation of a microenvironment that promotes tumor growth, metastasis, and angiogenesis (1, 2). Among its members, the 92-kDa type IV collagenase (MMP-9) is thought to be a key regulator of degradation of the type IV collagen component of basement membranes, and its expression and activity have been correlated with tumor progression (3). MMP-9 activity is regulated by several steps—at the level of gene transcription (4), mRNA stability (5), secretory process (6), and enzymatic activity. Gene expression of MMP-9 can be induced by a variety of stimuli, such as tumor necrosis factor α , epidermal growth factor (EGF), and phorbol esters (7, 8). Several transcription factors have been reported to activate the *MMP-9* promoter, indicating that MMP-9 expression is regulated by multiple mechanisms in tumor cells and contributes to tumor cell progression (9–11).

The reversion-inducing cysteine-rich protein with Kazal motifs (*RECK*) gene was isolated as an inducer of flat morphology of ν -K ras -transformed NIH3T3 cells (12). RECK expression is observed in human normal organs; however, several oncogenic factors, such as activated Ras (13, 14), EBV latent membrane protein 1 (15), and HER-2/neu (16), suppress the expression of RECK. It has been reported that overexpression of RECK decreases the amount of active MMP-2 and MMP-9 in conditioned medium and inhibits metastatic activity *in vitro* (12, 17, 18), and *in vivo* (19). Significant

correlation has been found between the abundance of RECK expression in hepatoma samples and colorectal cancer, and the survival of the patients (20, 21). Thus, RECK expression that plays a key role in the tumor metastasis can be a good prognostic indicator for cancer patients.

In this report, we show novel insight into RECK-mediated suppression of tumor cell invasion. Overexpression of RECK resulted in the decrease of *MMP-9* mRNA, and it was increased after treatment with RECK-specific siRNA in RECK-expressing cells. Promoter analysis and chromatin immunoprecipitation (ChIP) assay revealed that expression of RECK suppressed *MMP-9* promoter activity and decreased the direct interaction of Fra-1 and c-Jun to the 12-*O*-tetradecanoylphorbol-13-acetate-responsive element (TRE) within the *MMP-9* promoter region.

Materials and Methods

Cell culture. Human fibrosarcoma cells, HT1080, human normal lung fibroblast cells, WI-38, and human epidermoid carcinoma cells, A431, were cultured in DMEM (SIGMA) containing 10% FCS (SIGMA). RECK-over-expressing or mock-transfected HT1080 cells, designated as HT1080-RECK and HT1080-Zeo, respectively, were established by gene transfection using Effectene Transfection Reagent (QIAGEN) according to the manufacturer's instructions (18).

Plasmid construction. The human *RECK* gene was cloned into the pZeo-SV2 (+) vector (Invitrogen), as described before (18). The human *MMP-9* gene was cloned into the pcDNA3.1/Myc-His (+) vector (Invitrogen). The DNA fragment corresponding to 1024 bp of the 5'-flanking region of the human *MMP-9* promoter region (–1005 to +19) was amplified by PCR from genomic DNA of HT1080 and cloned into the pGL3-basic vector (Promega). A series of deletions and point mutants of putative *cis*-elements within the *MMP-9* promoter region were generated by PCR site-directed mutagenesis using the technique of overlap extension (22, 23). Mutant construction of putative *cis*-elements was designed according to previous reports: Smad-binding element (SBE; AGACAG to AtcgAG; ref. 24), κ B site (GGAATCCCC to GtcAacagCC; ref. 24), TRE-1 (TGAGTCA to TatGTCA; ref. 25), transforming growth factor- β inhibitory element (TIE; GGTTTGGGA to GGTcgGaGA; ref. 24), and TRE-2 (TGAGTCA to TttGTCA; ref. 4). The pRL-cytomegalovirus vector (Promega) was used to normalize transfection efficiency.

Western blotting. Cells were lysed in TENS buffer [10 mmol/L HEPES (pH 7.2), 142.5 mmol/L KCl, 5 mmol/L MgCl₂, 1 mmol/L EGTA, 0.2% Nonidet P-40, 0.1% aprotinin, and 2 mmol/L phenylmethylsulfonyl fluoride] and sonicated at 4°C. The lysates were centrifuged at 15,000 rpm for 15 min, and their protein concentration was adjusted by staining with Coomassie Brilliant Blue G-250 (Bio-Rad Laboratory). The 4 × sampling buffer [250 mmol/L Tris (pH 6.8), 40% glycerol, 8% SDS, 20% 2-mercaptoethanol, and 0.04% bromophenol blue] was added to each lysate before boiling them for 5 min. The samples were separated by SDS-polyacrylamide electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes (Millipore) and blotted with anti-RECK (611513; BD Bioscience), anti- α -tubulin (T9026; Sigma), or anti-c-Myc (sc-40; Santa Cruz) antibodies. For detection, enhanced chemiluminescence reagent (Pierce) was used with horseradish peroxidase-conjugated anti-mouse IgG (Bio-Rad Laboratory.) or anti-rabbit IgG secondary antibody (GE Healthcare).

Evaluation of MMP-9-MH secretion. Cells were seeded at 1.5×10^5 cells per well in 35-mm dishes (Falcon) for 18 h. After cell attachment,

Requests for reprints: Siro Simizu, Antibiotics Laboratory and Chemical Biology Department, Advanced Science Institute, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan. Phone: 81-48-467-9541; Fax: 81-48-462-4669; E-mail: simizu@riken.jp.
©2009 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-08-2635

the conditioned medium were exchanged for 1 mL of Opti-MEM (Invitrogen), and cells were transfected with pcDNA3.1-Neo or pcDNA3.1-MMP-9-MH with Effectene Transfection Reagent. This time point was defined as 0 h; cells and conditioned medium were harvested every 6 h. The sampling method of cell lysates has been described before in Western blotting. The cultured medium were centrifuged at 15,000 rpm for 15 min, and the supernatant was given 4× sampling buffer before boiling for 5 min.

Gelatin zymography. Cells were seeded at 1.0×10^5 cells per well in 24-well plates (Sumilon) for 12 h. After cell attachment, the conditioned medium were changed for 200 μ L of serum-free DMEM and incubated for a further 24 h. The cultured medium were collected and centrifuged at 15,000 rpm for 15 min, and 4× sampling buffer without 2-mercaptoethanol was added to supernatants. The samples were electrophoresed on an SDS-polyacrylamide gel containing 0.1% gelatin at 4°C. To remove SDS, the gel was washed with 2.5% Triton X-100 for 1 h and incubated with incubation buffer [50 mmol/L Tris (pH 7.5), 200 mmol/L NaCl, and 10 mmol/L CaCl₂] for 24 h at 37°C. Then, the gel was stained with Coomassie Brilliant Blue G-250 (26). In the case of the cell lysates, cells were washed with PBS and sonicated for 10 s after lysis without 2-mercaptoethanol sampling buffer at 4°C.

RNA interference. The pre-designed siRNAs that targeted *RECK* were purchased from Ambion (ID, 29149 for siRECK#1; ID, 29055 for siRECK#2; and ID, 29235 for siRECK#3). The siRNA duplex that was designed to target *GFP* (siGFP, 5'-GAACGGCAUCAAGGUGAACdTdT-3'), *Fra-1* (siFra-1, 5'-CACCAUGAGUGGCAGUCAGdTdT-3'), and *c-Jun* (siJun, 5'-GAUCCUGAAA-CAGAGCAUG-3') were obtained from Nippon EGT. The sequences of siRNA constructions refer to previous reports (27–29). Cells (1×10^5 cells) were seeded in 60-mm dishes and incubated for 12 h. Then, the cells were transfected with 200 pmol of annealed RNA using Lipofectamine2000 (Invitrogen) and incubated for 48 h.

Semiquantitative reverse transcription-PCR. The total RNA was isolated from cultured cells using ISOGEN (Nippon gene) according to the manufacturer's method, and a solution containing 5 μ g of total RNA was taken for the reverse transcription reaction that was performed with SuperScript 2 Reverse Transcriptase (Invitrogen) and oligo-dT primer. The sequences of the oligonucleotide primers were used as follows: *RECK* forward (5'-GCGGATGTCCGGGAACCTTGCCAG-3') and *RECK* reverse (5'-CTTCTTCTACAATGTTACCTAAAG-3'), *MMP-9* forward (5'-GGAGAC-CTGAGAACCAATCTC-3') and *MMP-9* reverse (5'-TCCAATAGGTGATG-TTGTCTG-3'), *MMP-2* forward (5'-TGTTGCGGCACCAGTGGACTAC-3') and *MMP-2* reverse (5'-TCAGCAGCCTAGCCAGTTCGGTTGATG-3'), and *GAPDH* forward (5'-TGAAGG TCGGAGTCAACGGATTTGGT-3') and *GAPDH* reverse (5'-CATGTGGCCATGAGGTCCACCAC-3'). PCR products were analyzed on a 2% agarose gel and visualized with ethidium bromide.

Quantitative real-time reverse transcription-PCR. The preparation of cDNA was as described above. SYBR Premix Ex Taq (TAKARA) was taken for quantitative real-time reverse transcription-PCR (RT-PCR), performed by the ABI 7500 Real-time PCR System (Applied Biosystems). The oligonucleotide primer pairs were designed as follows: *RECK* forward and (5'-ATCTGTT-CACCCCTGGAGT-3') *RECK* reverse (5'-TGTGAAGTGTGTTGCTTT-3'), *MMP-9* forward (5'-GCAGATTCCAAACCTTTGAG-3') and *MMP-9* reverse (5'-GCAAGTCTTCCGAGTAGT-3'), *MMP-2* forward (5'-CTCCCGAAAAGATT-GATG-3') and *MMP-2* reverse (5'-GGTGTGGCTGAGTAGAT-3'), *GAPDH* forward (5'-CTTACCACCATGGAGAAGGC-3') and *GAPDH* reverse (5'-GGCATGGACTGTGGTTCATGAG-3'). The amount of *GAPDH* RNA in each sample was determined from the calibration curve, and the level of each gene transcript was expressed relative to that of *GAPDH* mRNA (30).

Dual-luciferase reporter assay. The reporter assay was performed using the Dual-Luciferase Reporter Assay System (Promega). Cells were seeded on 24-well plates (5.0×10^4 cells), and after 12 h of incubation, cells were transfected with 400 ng of pGL3-MMP-9 plasmid vector and 1 ng of pRL-CMV vector using Lipofectamine2000 (Invitrogen). After a further 12 h, cultured medium was exchanged for 400 μ L of DMEM containing 10% FCS. After the next 12 h, cells were lysed in 250 μ L of the Passive Lysis Buffer, and 20 μ L of supernatant was used for the dual-luciferase reporter assay. The samples were counted for luminescence for 10 s in the 1420

ARVO Wallac (PerkinElmer), and the data were normalized by renilla luminescence.

ChIP assay. Cells were fixed with 1% formaldehyde for 15 min, washed, and lysed in ChIP lysis buffer [5 mmol/L HEPES (pH 8.0), 85 mmol/L KCl, and 0.5% NP40] at 4°C for 10 min. The nuclei were isolated by homogenization and then lysed in 140 μ L of nuclei lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 10 mmol/L EDTA, and 1% SDS]. To shear the DNA, the nuclei extracts were sonicated 8 times for 10 s with the Handy Sonic UR-20P (TOMY; SEIKO CO., LTD.). After sonication, the lysate was diluted 10-fold with ChIP dilution buffer [16.7 mmol/L Tris-HCl (pH 6.8), 167 mmol/L NaCl, 1.2 mmol/L EDTA, 0.01% SDS, and 1.1% Triton X-100] and centrifuged at 15,000 rpm for 15 min at 4°C (32). The supernatant protein concentration was adjusted and precleared by incubation with 100 μ L of Protein A suspension [30 mg of protein A sepharose CL-4B, 400 μ g of salmon sperm DNA (Invitrogen), and 1 mg bovine serum albumin (SIGMA) in 1 mL of TE buffer (pH 8.0)] for 120 min at 4°C (32). The supernatant was incubated with normal rabbit IgG (sc-2027; Santa Cruz), anti-c-Jun (sc-1694; Santa Cruz), anti-Jun B (sc-46; Santa Cruz), anti-Jun D (sc-74; Santa Cruz), anti-c-Fos (sc-52; Santa Cruz), anti-Fos B (sc-48; Santa Cruz), anti-Fra-1 (sc-605; Santa Cruz), or anti-Fra-2 (sc-604; Santa Cruz) antibodies at 4°C overnight. The immuno-complexes were then pulled down by 50 μ L of Protein A suspension. The sepharose beads were washed with wash buffer [0.1 mol/L sodium phosphate buffer (pH 6.8), containing 0.1% Tween 20] 4 times and given ChIP direct elution buffer [10 mmol/L Tris (pH 8.0), 300 mmol/L NaCl, 5 mmol/L EDTA, and 0.5% SDS], and the cross-links were reversed by heating at 65°C for 6 h. RNA was degraded with RNaseA (NACALAI TESQUE, INC.) at 37°C for 1 h, and protein was degraded with Proteinase K (NACALAI TESQUE, INC.) at 55°C for 1 h. DNA was purified with the QIAquick PCR Purification kit (QIAGEN) and used as template for PCR reactions. The primer pair was as follows: forward (5'-TACTGCCCTGAAGATTCAGC-3') and reverse (5'-TCCCAGGT-CAGATATCCTC-3').

GeneChip analysis. Total RNAs that were isolated from HT1080-Zeo and HT1080-RECK cells were converted to double-stranded cDNA with oligo-dT primers. The biotinylated cRNA, products of *in vitro* transcription with biotinylated UTP and CTP, were fragmented and hybridized on the Human Genome U133 Plus 2.0 Array (AFFYMETRIX). Data were normalized by *GAPDH* expression at the Research Resource Center of RIKEN Brain Science Institute for GeneChip hybridization services.

Statistics. The obtained values were expressed as the mean \pm SD and compared using Student's *t* test. In the figures, significant *P* values are shown as *P* < 0.05 (*).

Results

Overexpression of RECK decreases the level of MMP-9 mRNA. To clarify the molecular mechanism(s) of RECK-mediated suppression of tumor cell invasion, we first analyzed the gene expression profiles that were affected by RECK expression. Previously, we established RECK-overexpressing or mock-transfected HT1080 cells, designated as HT1080-RECK and HT1080-Zeo, respectively (Fig. 1A). We extracted mRNA from HT1080-RECK and HT1080-Zeo cells and compared mRNA expression. Interestingly, expression of *MMP-9* mRNA was decreased in HT1080-RECK cells (Fig. 1B). To confirm whether overexpression of RECK affects the amount of *MMP-9* mRNA, we compared the levels of mRNA expression by the real-time RT-PCR method. The level of *MMP-9* mRNA, but not *MMP-2* mRNA, was lower in HT1080-RECK cells than in HT1080-Zeo (Fig. 1C), indicating that overexpression of RECK results in the decrease of *MMP-9* mRNA, but not *MMP-2* mRNA, in HT1080 cells.

Correlation between RECK-mediated decrease of MMP-9 levels in cultured medium and expression of MMP-9 mRNA in mutant RECK-expressing cells. We previously established several

glycosylation-deficient mutants of RECK-expressing HT1080 cells and showed that Asn-297 was an important glycosylation residue in reducing MMP-9 in cultured medium (18). To examine whether RECK-mediated decrease of MMP-9 levels in cultured medium correlated with the levels of its mRNA in mutant RECK-expressing cells, we collected the conditioned medium and total RNAs from each cell line. Overexpression of wild-type RECK resulted in the decrease of both MMP-9 in cultured medium and *MMP-9* mRNA (Fig. 2), assessed by gelatin zymography and semiquantitative RT-PCR, respectively. Expression of RECK/N297Q, mutating Asn-297 to Gln; and RECK/4NQ, whereby all four Asn glycosylation residues, Asn-86, Asn-200, Asn-297, and Asn-352, were replaced with Gln; failed to reduce the levels of MMP-9 in conditioned medium and *MMP-9* mRNA (Fig. 2). On the other hand, expression of RECK/N86Q, changing Asn-86 to Gln; RECK/N200Q, changing Asn-200 to Gln; and RECK/N352Q, changing Asn-352 to Gln; reduced the level of MMP-9 in conditioned medium and *MMP-9* mRNA similar to the wild-type RECK expression level (Fig. 2). The level of *MMP-2* mRNA was not altered by the wild-type RECK or its mutants (Fig. 2). Therefore, these results strongly suggest that the decrease of MMP-9 in conditioned medium by RECK may be due to the reduction of *MMP-9* mRNA level.

Recovery of *MMP-9* mRNA expression by knockdown of RECK. Next, we examined the effect of RECK knockdown on the *MMP-9* mRNA levels using RECK-specific siRNAs (siRECK#1-3). siRECK#1 had the highest knockdown efficiency among the three siRNAs, and siRECK#3 had the lowest. Treatment of HT1080-RECK cells with siRECK#1-3 silenced the RECK expression, resulting in recovery of *MMP-9* mRNA levels, but not *MMP-2*, in a knockdown efficiency-dependent manner (Fig. 3A). Next, we treated two human cell lines, WI-38 (human lung fibroblast) and A431 (human

epidermoid carcinoma) cells, which express endogenous RECK with siRECK#1. The siRECK#1 treatment increased the level of *MMP-9* mRNA in both WI-38 and A431 cells (Fig. 3B). Taken together, these results suggest that RECK is a novel regulator of *MMP-9* mRNA.

RECK decreases MMP-9 in culture medium due to the decrease of *MMP-9* mRNA levels. Previous reports showed that a recombinant RECK protein, which has 23 amino acids deleted from its COOH terminal, bound to purified pro-MMP-9 *in vitro*. It has been indicated that RECK-mediated decrease of MMP-9 in cultured medium can be due to (a) RECK interaction with MMP-9 on the cell surface, and/or (b) RECK indirectly influencing MMP-9 secretion (12). Thus, we examined whether RECK affects MMP-9 secretion. We first detected the amount of both intracellular and extracellular MMP-9. If RECK suppresses MMP-9 secretion, the accumulation of intracellular MMP-9 would be observed in HT1080-RECK cells. We found that, the amount of intracellular MMP-9 was too low to be detected in HT1080-RECK cells (Fig. 4A). Moreover, we transfected the expression vector that encoded Myc-His-tagged MMP-9 (MMP-9-MH) into HT1080-Zeo and HT1080-RECK cells, and monitored the amount of secreted MMP-9-MH into the cultured medium every 6 h. During the time course experiment, the amount of secreted MMP-9-MH was similar in both HT1080-Zeo and HT1080-RECK cells (Fig. 4B). This result suggests that RECK-mediated decrease of MMP-9 in the culture medium may be mainly due to the decrease in *MMP-9* mRNA levels.

Suppression of *MMP-9* promoter activity by RECK. Because the level of *MMP-9* mRNA was decreased in HT1080-RECK, we next examined the effect of RECK expression on *MMP-9* promoter activity. We cloned the human *MMP-9* promoter region (–1005 to

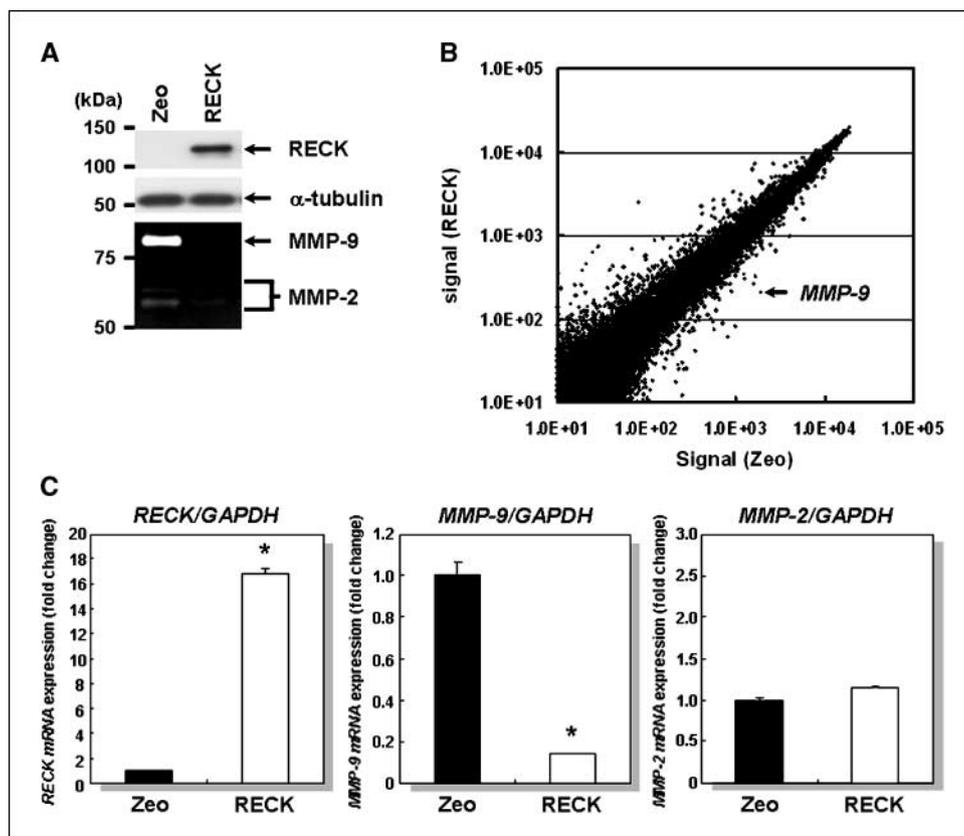


Figure 1. Decrease of *MMP-9* mRNA by RECK. *A*, establishment of HT1080-Zeo and HT1080-RECK cells. The cells were washed and cultured in serum-free medium for 24 h and lysed, and aliquots of cell lysates were immunoblotted with the indicated antibodies (*top*). The prepared conditioned medium from each cell line were analyzed by gelatin zymography (*bottom*). *B*, GeneChip analysis of RECK-overexpressing cells. The cRNA fragments prepared from HT1080-Zeo and HT1080-RECK cells were hybridized to Affymetrix Human Genome U133 Plus 2.0 arrays, and the gene expression profiles were compared. *C*, expression of *MMP-9* and *MMP-2* mRNAs in HT1080-Zeo and HT1080-RECK cells. Total RNAs were isolated from HT1080-Zeo and HT1080-RECK cells, and real-time RT-PCR assay were performed. Expression levels were normalized by *GAPDH* mRNA. The error bars indicate mean \pm SD of triplicate experiments. *, $P < 0.05$ compared with HT1080-Zeo cells.

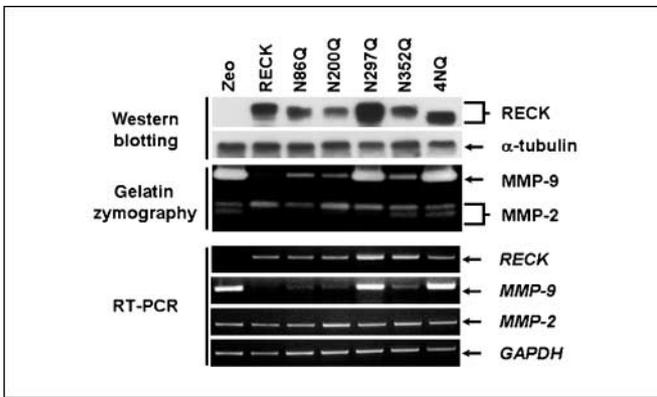


Figure 2. Effect of mutant RECK expression on the expression level of *MMP-9* mRNA. HT1080-Zeo, HT1080-RECK, HT1080-RECK/N86Q, HT1080-RECK/N200Q, HT1080-RECK/N297Q, HT1080-RECK/N352Q, and HT1080-RECK/4NQ cells were washed and cultured in serum-free medium for 24 h. Cells were lysed, and aliquots of cell lysates were immunoblotted with the indicated antibodies (*Western blotting*). The prepared conditioned medium from each cell line was analyzed by gelatin zymography (*gelatin zymography*), and total RNAs were isolated from each cell line and semiquantitative RT-PCR was performed (*RT-PCR*).

+19) into the pGL3 basic vector, designated as pGL3-MMP-9 (−1000). There were several putative transcription factor-binding sites in this region, analyzed by the Transcription Element Search Software³ (Fig. 5A). This vector and the phRL-CMV vector were transiently transfected in HT1080-Zeo and HT1080-RECK cells, and the promoter activity of *MMP-9* was measured by luciferase luminescence. Our results indicate that, ~60% of *MMP-9* promoter activity was suppressed in HT1080-RECK cells compared with HT1080-Zeo cells (Fig. 5B), suggesting RECK expression suppresses *MMP-9* promoter activity. Furthermore, to identify the region(s) in the *MMP-9* promoter that is required for the suppression of *MMP-9* promoter activity by RECK, we generated several deletion mutants of the *MMP-9* promoter region. The vectors that contained the promoter region −700 to +19, −400 to +19, and −100 to +19 were designated as pGL3-MMP-9 (−700), pGL3-MMP-9 (−400), and pGL3-MMP-9 (−100), respectively (Fig. 5B). RECK-mediated suppression of promoter activity that were transfected with pGL3-MMP-9 (−700) was similar to that of pGL3-MMP-9 (−1000); however, it was not observed in cells that were transfected with pGL3-MMP-9 (−400) or pGL3-MMP-9 (−100; Fig. 5B). These results indicate that the *MMP-9* promoter region that is located at −700 to −400 bp is the important region for RECK-mediated suppression of *MMP-9* promoter activity.

To further analyze the contribution of this region (−700 to −400 bp), we constructed more vectors that were mutated at putative transcription factor-binding sites within the *MMP-9* promoter, −620/−615, −600/−591, −533/−527, −474/−465, and −79/−73, designated as pGL3-MMP-9 (mSBE), pGL3-MMP-9 (mκB), pGL3-MMP-9 (mTRE-1), pGL3-MMP-9 (mTIE), and pGL3-MMP-9 (mTRE-2), respectively, and each promoter activity was examined in HT1080-Zeo and HT1080-RECK cells. We found that RECK-mediated suppression of *MMP-9* promoter activity was observed in pGL3-MMP-9 (mSBE)−, pGL3-MMP-9 (mTIE)−, and pGL3-MMP-9 (mTRE-2)−transfected cells; however, in pGL3-MMP-9 (mκB)−transfected cells, the promoter activity was similar in the presence

or absence of RECK, and in pGL3-MMP-9 (mTRE-1)−transfected cells, the difference is significant but the suppression was less compared with the other mutants (Fig. 5C). These results suggest that κB site and TRE-1 within the *MMP-9* promoter region are important for RECK-mediated suppression of *MMP-9* promoter activity.

Binding of Fra-1 and c-Jun to TRE-1 within the *MMP-9* promoter region is suppressed by RECK. To determine whether RECK influences the binding of activator protein to the TRE-1, ChIP assay was performed. The protein-DNA complexes were immunoprecipitated with anti-Fos family (c-Fos, Fos B, Fra-1, and Fra-2) and anti-Jun family (c-Jun, Jun B, and Jun D) antibodies, followed by PCR reactions that targeted TRE-1 containing region (Fig. 6A). The samples of HT1080-Zeo cells that were immunoprecipitated with anti-Fra-1 and anti-c-Jun antibodies showed the PCR bands, suggesting that these transcription factors might bind to TRE-1 within the *MMP-9* promoter region. However, the interactions of Fra-1 and c-Jun with TRE-1 were suppressed in HT1080-RECK cells (Fig. 6B). Although the bindings of the nuclear factor-κB (NF-κB) components p65 and p50 to the κB site were examined, we could not detect the bindings (data not shown). The protein expression levels of Jun family proteins, Fos family proteins (Fig. 6C), and NF-κB components (data not shown) in nuclei were the same for both HT1080-Zeo and HT1080-RECK cells. Therefore, these results indicate that RECK suppresses the binding of Fra-1 and c-Jun to TRE-1 within the *MMP-9* promoter region. Finally, we sought to investigate whether Fra-1 and c-Jun regulate *MMP-9* mRNA levels. Treatment with siRNAs that targeted *Fra-1* (siFra-1) and *c-Jun* (sijun) of HT1080-

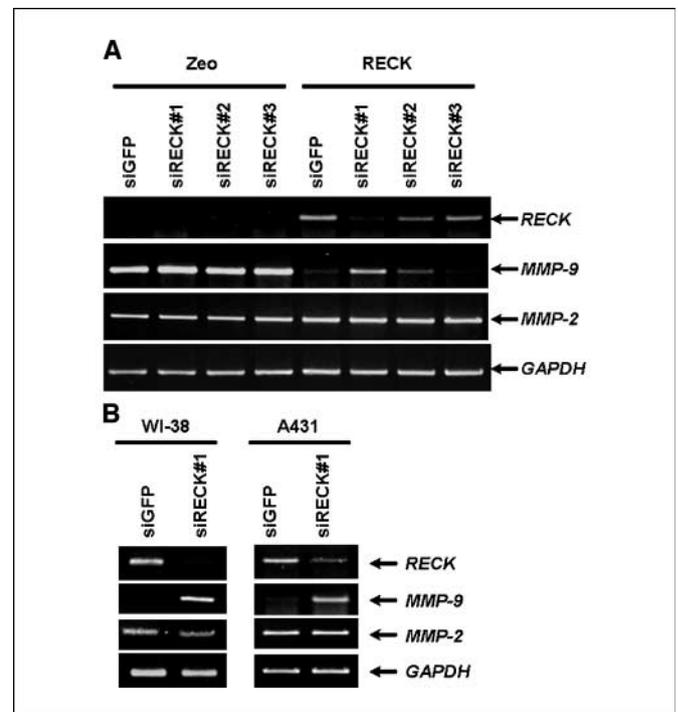


Figure 3. Effect of RECK-specific siRNA on the amount of *MMP-9* mRNA. **A**, effect of RECK-specific siRNA (*siRECK#1-3*) on *MMP-9* mRNA in HT1080-Zeo and HT1080-RECK cells. Cells were treated with GFP siRNA (*siGFP*) as a control siRNA or *siRECK#1-3*. Then, the expression of mRNA levels was analyzed by semiquantitative RT-PCR. **B**, effect of *siRECK#1* on *MMP-9* mRNA in WI-38 and A431 cells. WI-38 (*left*) and A431 (*right*) cells were treated with *siGFP* or *siRECK#1*, and the expression of mRNA levels was measured by semiquantitative RT-PCR.

³ <http://www.cbil.upenn.edu/cgi-bin/tess/tess>

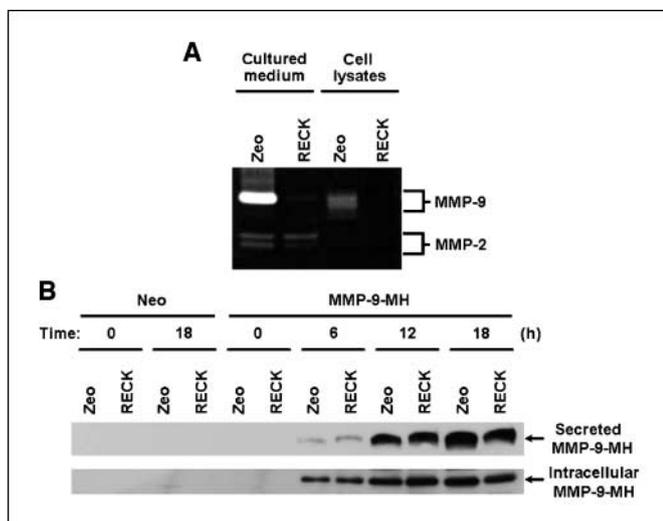


Figure 4. Effect of RECK expression on the secretion of MMP-9. *A*, effect of RECK expression on the amounts of both extracellular and intracellular MMP-9. HT1080-Zeo and HT1080-RECK cells were washed and cultured in serum-free medium for 24 h. The prepared conditioned medium and cell lysates from each cell line were analyzed by gelatin zymography. *B*, effect of RECK expression on the secretion of exogenous MMP-9. HT1080-Zeo and HT1080-RECK cells were transfected with pcDNA 3.1-MH or pcDNA3.1-MMP-9-MH vector, and the amounts of secreted MMP-9-MH in cultured medium and that of intracellular MMP-9-MH levels were detected every 6 h by Western blotting using anti-Myc antibody.

Zeo cells resulted in the decrease of *MMP-9* mRNA (Fig. 6D). To exclude the possibility of off-target effects, we also detected the levels of *Jun D* and *Fra-2* expression, these genes were not affected by the siRNA silencing (data not shown). Thus, taken together, these results indicate that Fra-1 and c-Jun contribute to the regulation of *MMP-9* mRNA level and RECK decreases the binding of Fra-1 and c-Jun to the *MMP-9* promoter region, thereby inhibiting *MMP-9* mRNA expression.

Discussion

Proteases and glycosidases are essential components in both physiologic and pathologic turnover of ECM by degrading macromolecules, and disruption of their regulatory balance has been observed during tumor cell metastasis processes (1–4, 26). The activity of MMP is regulated at multiple steps, including gene transcription, mRNA stability, protein secretion, and enzymatic activity by inhibitory proteins (4–6). Although RECK functions as a negative regulator of MMP by inhibiting enzymatic activities and secretion (12, 18), the molecular mechanism(s) of RECK-mediated suppression of tumor cell invasion has not yet been clarified.

In this study, we performed GeneChip analysis and showed a decrease of *MMP-9* mRNA level in RECK-expressing cells (Fig. 1B). We also checked the levels of some metastasis-associated genes, such as other MMPs, glycosidases, cell adhesion molecules, and proteinase inhibitors, comparing HT1080-Zeo with HT1080-RECK cells; however, we could not find any metastasis-associated genes that were down-regulated by the same degree as *MMP-9* mRNA (data not shown). On the other hand, we could find some genes whose expressions were changed by RECK expression, such as Cullin-2 or KIF21A. Further studies are needed to examine the role of these genes in RECK-mediated suppression of tumor cell invasion.

It has been proposed that RECK can bind to *MMP-9* *in vitro*; thus, the mechanism of decreasing the *MMP-9* levels in culture

medium by RECK might be due to the inhibition of *MMP-9* secretion on the cell membrane (12, 33). However, in this report, we have shown that overexpression of RECK results in the decrease of *MMP-9* mRNA (Figs. 1 and 2), and the knockdown of RECK expression recovers *MMP-9* mRNA levels in both exogenous and endogenous RECK-expressing cells (Fig. 3). Moreover, the amounts of intracellular and extracellular *MMP-9* were hardly detected in HT1080-RECK cells (Fig. 4A). Furthermore, RECK did not inhibit the secretion of exogenously expressed *MMP-9* (Fig. 4B). Thus, we concluded that the decrease of *MMP-9* level in the cultured medium by RECK expression was mainly due to the inhibition of *MMP-9* mRNA level rather than suppression of *MMP-9* secretion. Therefore, our data indicate that RECK is a novel type of negative regulator of *MMP-9* mRNA.

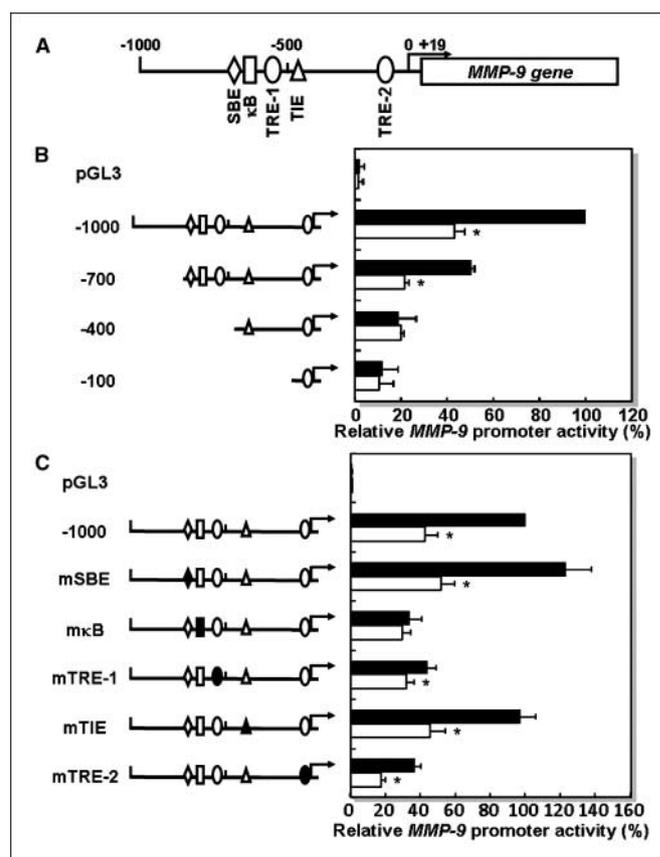


Figure 5. Suppression of *MMP-9* promoter activity by RECK. *A*, an illustration of the putative transcription factor-binding sites (diamond, SBE; rectangle, κ B; ellipses, TRE-1 and TRE-2; triangle, TIE) within the *MMP-9* promoter region. *B*, effect of RECK expression on *MMP-9* promoter activity. HT1080-Zeo (closed bars) and HT1080-RECK (open bars) cells were transfected with the control vector (pGL3), the plasmid containing the full-length *MMP-9* promoter region (–1000), or the deletion constructs (–700, –400, or –100) with pRL-CMV vector (encoded renilla luciferase; used for normalization of transfection efficiencies), and the luciferase activities were measured after 24 h of transfection. The promoter activity of HT1080-Zeo cells transfected with –1000 was defined as 100%. Columns, means of three independent experiments; bars, SD. *C*, effect of mutations at the *cis* elements within the *MMP-9* promoter region on RECK-mediated suppression of *MMP-9* promoter activity. The putative transcription factor-binding sites within the *MMP-9* promoter region were mutated (closed diamond, closed square, closed ellipses, or closed triangle). HT1080-Zeo (closed bars) and HT1080-RECK (open bars) cells were cotransfected with the mutant constructs and pRL-CMV vector, and the luciferase activities were measured 24 h after transfection. The promoter activity of HT1080-Zeo cells transfected with –1000 was defined as 100%. Columns, mean of 6 independent experiments; bars, SD. *, $P < 0.05$ compared with HT1080-Zeo cells.

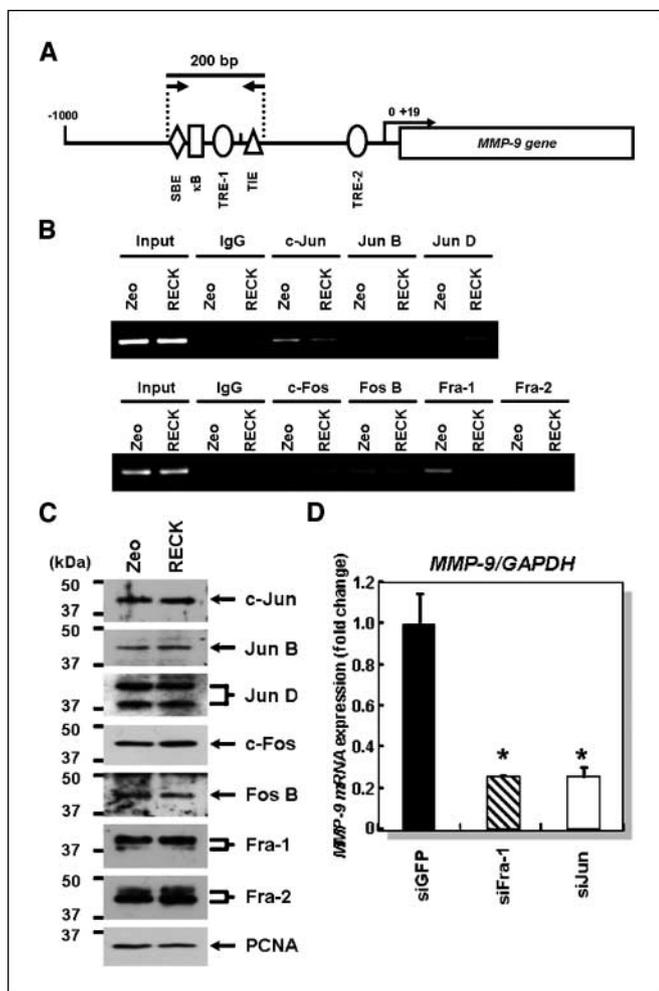


Figure 6. RECK inhibits the binding of Fra-1 and c-Jun to TRE-1 within the *MMP-9* promoter region. **A**, an illustration of the *MMP-9* promoter region. The locations of the primer set used for ChIP assay are indicated. **B**, effect of RECK expression on the binding of AP-1 components to the *MMP-9* promoter region. HT1080-Zeo and HT1080-RECK cells were fixed and lysed, and aliquots of cell lysates were immunoprecipitated with the indicated antibodies. The resultant samples were used as templates for PCR. **C**, the expression levels of AP-1 components in nuclear extracts of HT1080-Zeo and HT1080-RECK cells. HT1080-Zeo and HT1080-RECK cells were homogenized, and nuclei were isolated. Aliquots of nuclei extracts were immunoblotted with the indicated antibodies. **D**, effects of siRNAs targeting to *Fra-1* and *c-Jun* on *MMP-9* mRNA expression. The cells were treated with siRNAs targeting *Fra-1* (*siFra-1*) and *c-Jun* (*siJun*), and the gene expression levels were detected by real-time RT-PCR. Columns, mean of triplicate experiments; bars, SD. *, $P < 0.05$ compared with siGFP-treated HT1080-Zeo cells.

MMP-9 is well-characterized as a key promoter of tumor cell invasion (1). We observed that treatment with RECK-specific siRNA of WI-38 cells increases the *MMP-9* mRNA level (Fig. 3B); however, it does not make cells invasive (data not shown). Our result may show the possibility that *MMP-9* is necessary but not sufficient for the invasive phenotype. Further study is needed to delineate the exact mechanism.

Our luciferase reporter assay showed that RECK suppresses *MMP-9* promoter activity through the κ B and TRE-1 sites (Fig. 5C). There are mounting evidences that *MMP-9* expression is regulated by AP-1 and NF- κ B (4, 7, 8, 25, 34). As for the TRE-1 site, we found Fra-1 and c-Jun as *MMP-9* promoter-binding transcriptional factors (Fig. 6B); however, we could not detect any binding of NF- κ B components within the *MMP-9* promoter region by ChIP analysis. Thus, we speculated that the κ B site is important

for RECK-mediated suppression of *MMP-9* transcription, but unidentified transcription factor(s) might bind and regulate *MMP-9* mRNA expression through the κ B site. Moreover, overall activity is suppressed in mTRE-2 which is located just upstream of the TATA-box, we thought that mutation of TRE-2 may influence the recruitment of basal transcription factors. Further studies are necessary to resolve these phenomena.

We showed that RECK decreased the binding of Fra-1 and c-Jun to TRE-1 within the *MMP-9* promoter region (Fig. 6B), and that treatment with siRNAs that targeted *Fra-1* and *c-Jun* decrease the *MMP-9* mRNA level (Fig. 6D). In a previous study, Fra-1-overexpressing cells represented the up-regulation of *MMP-9* gene expression by microarray experiments, and the activity of the *MMP-9* promoter was increased by Fra-1 expression (35). Another group showed that Fra-1 and Stat3 synergistically regulated the expression of human *MMP-9* mRNA (36). These reports indicate that Fra-1 contributes to the up-regulation of *MMP-9* transcription. On the other hand, Fra-1 is also known to be accumulated in *ras*-transformed NIH3T3 fibroblasts (37), and the accumulation in both transcription and protein stabilization level is mediated by the MAP/ERK kinase/extracellular signal-regulated kinase pathway (28, 38). Stable transfection with a Fra-1 antisense RNA vector significantly reduces the malignant phenotype in *ras*-transformed FRTL-5 thyrocytes, indicating that Fra-1 plays crucial roles in *ras*-induced transformation (39, 40). Interestingly, RECK was originally identified as a suppressor gene of *v-Ki-ras*-transformed NIH3T3 cells, and our findings show that RECK suppresses Fra-1 activity, suggesting that reversion of *ras*-transformed NIH3T3 cells by RECK might be mediated by suppression of Fra-1 activity.

Because RECK is a glycosylphosphatidylinositol-anchored protein with no transmembrane domain or intracellular domain, the mechanism to suppress Fra-1 and c-Jun bindings to the *MMP-9* promoter region is very interesting. Recently, it has been reported that ErbB-2, a member of the EGF receptor tyrosine kinase family, translocates into the nucleus and binds to the *COX-2* promoter to enhance transcriptional activity (31). To examine whether RECK is translocated into the nucleus, we performed cell fractionation experiments and immunohistochemistry with anti-RECK antibody; however, we could not obtain positive evidence that RECK might be localized in the nuclear (data not shown). At present, we speculate that RECK might weaken signal transduction by interacting with some receptors at the plasma membrane and thus leads to the decrease in binding of Fra-1 and c-Jun to the *MMP-9* promoter region.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 7/9/2008; revised 11/19/2008; accepted 12/3/2008.

Grant support: A Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Junior Research Associate fellowship of RIKEN (S. Takagi).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Drs. M. Noda, C. Takahashi, M. G. Suzuki, Y. Tamura, and T. Miki for their valuable suggestions; K. Maola and E. Ong for critical comments on manuscript; Y. Ichikawa and R. Nakazawa (Bioarchitect Research Group, RIKEN) for the DNA sequencing; and the members of the Research Resource Center at the RIKEN Brain Science Institute for GeneChip hybridization services.

References

1. Bergers G, Brekken R, McMahon G, et al. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat Cell Biol* 2000;2:737-44.
2. Jodele S, Chantrain CF, Blavier L, et al. The contribution of bone marrow-derived cells to the tumor vasculature in neuroblastoma is matrix metalloproteinase-9 dependent. *Cancer Res* 2005;65:3200-8.
3. Bernhard EJ, Gruber SB, Muschel RJ. Direct evidence linking expression of matrix metalloproteinase 9 (92 kDa gelatinase/collagenase) to the metastatic phenotype in transfected rat embryo cells. *Proc Natl Acad Sci U S A* 1994;91:4293-7.
4. Sato H, Seiki M. Regulatory mechanism of 92 kDa type IV collagenase gene expression which is associated with invasiveness of tumor cells. *Oncogene* 1993;8:395-405.
5. Iyer V, Pumiqlia K, DiPersio CM. $\alpha 3\beta 1$ integrin regulates MMP-9 mRNA stability in immortalized keratinocytes: a novel mechanism of integrin-mediated MMP gene expression. *J Cell Sci* 2005;118:1185-95.
6. Ramos DN, Hahn DE, Siple J, et al. Activation of matrix metalloproteinase-9 (MMP-9) via a converging Plasmin/Stromelysin-1 cascade enhances tumor cell invasion. *J Biol Chem* 1999;274:13066-76.
7. Watanabe H, Nakanishi I, Yamashita K, Hayakawa T, Okada Y. Matrix metalloproteinase-9 (92 kDa gelatinase/type IV collagenase) from U937 monoblastoid cells: correlation with cellular invasion. *J Cell Sci* 1993;104:991-9.
8. Lyons JG, Birkedal HB, Pierson MC, Whitelock JM, Birkedal HH. Interleukin-1 β and transforming growth factor- α /epidermal growth factor induce expression of M(r) 95,000 type IV collagenase/gelatinase and interstitial fibroblast-type collagenase by rat mucosal keratinocytes. *J Biol Chem* 1993;268:19143-51.
9. Ma Z, Shah RC, Chang MJ, Benveniste EN. Coordination of cell signaling, chromatin remodeling, histone modifications, and regulator recruitment in human matrix metalloproteinase 9 gene transcription. *Mol Cell Biol* 2004;24:5496-509.
10. Kobayashi T, Kishimoto J, Ge Y, et al. A novel mechanism of matrix metalloproteinase-9 gene expression implies a role for keratinization. *EMBO Rep* 2001;2:604-8.
11. Schwartz B, Melnikova VO, Tellez C, et al. Loss of AP-2 α in deregulation of E-cadherin and MMP-9 and an increase in tumorigenicity of colon cancer cells *in vivo*. *Oncogene* 2007;26:4049-58.
12. Takahashi C, Sheng Z, Horan TP, et al. Regulation of matrix metalloproteinase-9 and inhibition of tumor invasion by the membrane-anchored glycoprotein RECK. *Proc Natl Acad Sci U S A* 1998;95:13221-6.
13. Sasahara RM, Takahashi C, Noda M. Involvement of the Sp1 site in ras-mediated down-regulation of the RECK metastasis suppressor gene. *Biochem Biophys Res Commun* 1999;264:668-75.
14. Chang HC, Liu LT, Hung WC. Involvement of histone deacetylation in ras-induced down regulation of metastasis suppressor RECK. *Cell Signal* 2004;16:675-9.
15. Liu LT, Peng JP, Chang HC, Hung WC. RECK is a target of Epstein-Bar virus latent membrane protein 1. *Oncogene* 2003;22:8263-70.
16. Hsu MC, Chang HC, Hung WC. HER-2/neu represses the metastasis suppressor RECK via ERK and Sp transcription factor to promote cell invasion. *J Biol Chem* 2006;281:4718-25.
17. Oh J, Takahashi R, Kondo S, et al. The membrane-anchored MMP inhibitor RECK is a key regulator of extracellular matrix integrity and angiogenesis. *Cell* 2001;107:789-800.
18. Simizu S, Takagi S, Tamura Y, Osada H. RECK-mediated suppression of tumor cell invasion is regulated by glycosylation in human tumor cell lines. *Cancer Res* 2005;65:7455-61.
19. Chang CK, Hung WC, Chang HC. The Kazal motifs of RECK protein inhibit MMP-9 secretion and activity and reduce metastasis of lung cancer cells *in vitro* and *in vivo*. *J Cell Mol Med* 2008; ahead of print.
20. Furumoto K, Arai S, Mori A, et al. RECK gene expression in hepatocellular carcinoma, Correlation with invasion-related clinicopathological factors and its clinical significance. *Hepatology* 2001;33:189-95.
21. Takeuchi T, Hisanaga M, Nagao M, et al. The membrane-anchored matrix metalloproteinase (MMP) regulator RECK in combination with MMP-9 serves as an information prognostic indicator for colorectal cancer. *Clin Cancer Res* 2004;10:5572-9.
22. Teruya T, Simizu S, Kanoh N, Osada H. Phosphatase 2A catalytic subunit in cells. *FEBS Lett* 2005;579:2463-8.
23. Tamura Y, Simizu S, Osada H. The phosphorylation status and anti-apoptotic activity of Bcl-2 are regulated by ERK and protein phosphatase 2A on the mitochondria. *FEBS Lett* 2004;569:249-55.
24. Ogawa K, Chem F, Kuang C, Chem Y. Suppression of matrix metalloproteinase-9 transcription by transforming growth factor- β is mediated by nuclear factor- κ B site. *Biochem J* 2004;381:413-22.
25. Gum R, Lengyel E, Juarez J, et al. Stimulation of 92-kDa gelatinase B promoter activity by ras is mitogen-activated protein kinase 1-independent and requires multiple transcription factor binding sites including closely spaced PEA/*ets* and AP-1 sequences. *J Biol Chem* 1996;271:10672-80.
26. Simizu S, Suzuki T, Muroi M, et al. Involvement of disulfide bond formation in the activation of heparinase. *Cancer Res* 2007;67:7841-9.
27. Dubois T, Paleotti O, Mironov AA, et al. Golgi-localized GAP for Cdc42 functions downstream of ARF1 to control Arp2/3 complex and F-actin dynamics. *Nat Cell Biol* 2005;7:353-63.
28. Vial E, Marshall CJ. Elevated ERK-MAP kinase activity protects the FOS family member FRA-1 against proteasomal degradation in colon carcinoma cells. *J Cell Sci* 2003;116:4957-63.
29. Holmström TH, Mialon A, Kallio M, et al. c-Jun supports ribosomal RNA processing and nucleolar localization of RNA helicase DDX21. *J Biol Chem* 2008;283:7046-53.
30. Koike Y, Mita K, Suzuki MG, et al. Genomic sequence of a 320-kb segment of the Z chromosome of *Bombyx mori* containing a *kettin* ortholog. *Mol Gen Genomics* 2003;269:137-49.
31. Wang SC, Lien HC, Xia W, et al. Binding at and transactivation of the COX-2 promoter by nuclear tyrosine kinase receptor ErbB-2. *Cancer Cell* 2004;6:251-61.
32. Ma Z, Shah RC, Chang MJ, Benveniste EN. Coordination of cell signaling, chromatin remodeling, histone modifications, and regulator recruitment in human matrix metalloproteinase 9 gene transcription. *Mol Cell Biol* 2004;24:5496-509.
33. Chang CK, Hung WC, Chang HC. The Kazal motifs of RECK protein inhibit MMP-9 secretion and activity and reduce metastasis of lung cancer cells *in vitro* and *in vivo*. *J Cell Mol Med*. In press.
34. Yokoo T, Kitamura M. Dual regulation of IL-1 β -mediated matrix metalloproteinase-9 expression in mesangial cells by NF- κ B and AP-1. *Am J Physiol* 1996;270:123-30.
35. Belguise K, Kersual N, Galtier F, Chalbos D. Fra-1 expression level regulates proliferation and invasiveness of breast cancer cells. *Oncogene* 2005;24:1434-44.
36. Song Y, Qian L, Song S, et al. Fra-1 and Stat3 synergistically regulate activation of human MMP-9 gene. *Mol Immunol* 2008;45:137-43.
37. Mehta F, Lallemand D, Pfarr CM, Yaniv M. Transformation by ras modifies AP1 composition and activity. *Oncogene* 1997;14:837-47.
38. Casalino L, De Cesare D, Verde P. Accumulation of Fra-1 in ras-transformed cells depends on both transcriptional autoregulation and MEK-dependent posttranslational stabilization. *Mol Cell Biol* 2003;23:4401-15.
39. Vallone D, Battista S, Pierantoni GM, et al. Neoplastic transformation of rat thyroid cells requires the junB and fra-1 gene induction which is dependent on the HMGI-C gene product. *EMBO J* 1997;16:5310-21.
40. Casalino L, Bakiri L, Talotta F, et al. Fra-1 promotes growth and survival in RAS-transformed thyroid cells by controlling cyclin A transcription. *EMBO J* 2007;26:1878-90.