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Molecular Cloning of Canine Membrane-Anchored Inhibitor of Matrix Metalloproteinase, RECK

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ABSTRACT. The reversion-inducing cysteine-rich protein with Kazal motifs (*RECK*) gene is one of the endogenous matrix metalloproteinase (MMP) inhibitors. It was reported that decreased *RECK* expression closely correlated with tumor malignancy. We determined the cDNA sequence of the canine *RECK* gene. The cDNA sequence and deduced amino acid of canine *RECK* were 2,913 bases and 971 residues, respectively. The predicted amino acid sequence of the protein showed 95.5% and 91.9% homology with human and mouse *RECK*, respectively. *RECK* mRNA expression was analyzed in various canine tissues and tumor cell lines by quantitative RT-PCR. The highest *RECK* expression was detected in lung and testis. In comparison with the tissues, a remarkably low expression level was detected in tumor cell lines. In addition, the *RECK* gene was transfected in the canine transitional cell carcinoma, and its influence on cell proliferation, migration, and invasion was analyzed. The transfected *RECK* gene suppressed only canine tumor invasion. These results showed that *RECK* might play an important role in tumor malignancy in dogs as well as in other mammalians.

KEY WORDS: canine, extracellular matrix, proteinase inhibitor, RECK, tumor malignancy.

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Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that selectively degrade the extracellular matrix. The MMP family consists of at least 20 enzymes divided into five different groups: collagenases, gelatinases, stromelysins, membrane-type MMPs, and other MMPs. MMPs, particularly those from the gelatinase group, MMP-2 (72 kDa, Gelatinase A) and MMP-9 (92 kDa, Gelatinase B), appear to play an important role in cancer pathology, especially in invasion, metastasis, and angiogenesis [6, 10, 20].

The reversion-inducing cysteine-rich protein with Kazal motifs (*RECK*) gene was isolated by cDNA expression cloning as a gene that induces a flat morphology when expressed in the v-Ki-ras-transformed NIH 3T3 cell line [21]. RECK was reported as an endogenous MMP inhibitor and a membrane-anchored glycoprotein (approximately 110 kDa) with multiple epidermal growth factor-like repeats and serine protease inhibitor-like domains. *RECK* negatively regulates MMP-2, MMP-9, and membrane-type matrix metalloproteinase (MT1-MMP) [16, 21]. Although *RECK* mRNA was expressed in most normal human cells and tissues, it was undetectable in many tumor cell lines and in cells artificially expressing active oncogenes [21]. In addition, clinical studies also indicated that patients with high *RECK* expression in tumor tissues showed better survival, and such tumors were less invasive [7, 19].

In veterinary medicine too, cancer is one of the major fatal diseases, and spontaneously developed tumors in dogs are very similar to those in humans [5, 15]. Therefore, it is expected that *RECK* gene may play an important role in tumor malignancy in dogs as in the case of humans.

The purpose of the present study was to determine the cDNA sequence of canine endogenous MMP inhibitor, *RECK*. We studied *RECK* mRNA distribution in various canine tissues and tumor cell lines and analyzed the influence of transfected *RECK* on tumor cell lines.

MATERIALS AND METHODS

Cloning and sequencing of canine RECK cDNA: Total RNA was extracted from testes obtained from two adult healthy male beagles by the guanidine isothiocyanate method [4]. To obtain canine *RECK* cDNA, the total RNA was reverse-transcribed using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) and oligo(dT)-primer, and PCR amplification was performed with *Taq* DNA polymerase (Perkin Elmer, Tokyo, Japan) using primer pairs (Table 1) designed using previously reported sequences of human and mouse *RECK* cDNAs. To amplify the 3' end, cDNA was amplified by rapid amplification of 3' cDNA ends (3'RACE) procedure. Reverse-transcription of the 5' end of cDNA was performed at a high temperature (64°C) using Thermo Script RT (Invitrogen) and canine *RECK* gene specific primer [17].

The PCR products were cloned into pGEM T-Easy vector (Promega, Madison, WI, U.S.A.), and the nucleotide sequences were determined by the cycle-sequence method using a DNA sequencer (ABI PRISM 310 Genetic analyzer; Perkin Elmer, Wellesley, MA, U.S.A.). At least two colonies were cloned for each dog. If all the clones did not have the same sequence, minority clones were accepted as PCR errors. To confirm that this sequence was cloned by single cDNA, we also checked whether the full length of cDNA could be amplified without being divided into short sec-

Table 1. Sequences of oligonucleotide primers

Primers	Primer Sequences	Purpose
hF1	5'-AAGCTGGGTCCGAGCATCCC-3'	For amplifying canine *RECK* cDNA 1
cR1	5'-CAACACAGTGCACCCGCGCT-3'	For amplifying canine *RECK* cDNA 1
hF2	5'-TGCGCTGCTCCTTCTGCT-3'	For amplifying canine *RECK* cDNA 2
cR2	5'-CTGGGGCTCTCTGTAACAGATGC-3'	For amplifying canine *RECK* cDNA 2
hF3	5'-GATAACCAAATGTGCCGTGATGC-3'	For amplifying canine *RECK* cDNA 3
cR3	5'-GTGATGGCCTGCATAACTGC-3'	For amplifying canine *RECK* cDNA 3
mF4	5'-GCATGCAAGCAGGCATCTTC-3'	For amplifying canine *RECK* cDNA 4
mR4	5'-TTGTAAGACCCAGCCCTTGC-3'	For amplifying canine *RECK* cDNA 4
cF5	5'-GCTGAAGACCATGCTTGCCA-3'	For amplifying canine *RECK* cDNA 5
cR5	5'-CAACCAACTTCCCCTGCAGA-3'	For amplifying canine *RECK* cDNA 5, For real time PCR
cF6	5'-CCTTGCCAGTTGGGCTGTAG-3'	For amplifying canine *RECK* cDNA 6
cR6	5'-ATGCAGGCACTGGGGTAAGT-3'	For amplifying canine *RECK* cDNA 6
cF7	5'-ACAGGTCTGCCCTGTAACTTG-3'	For amplifying canine *RECK* cDNA 7
mR7	5'-GGTGGGATGATGGGTTTGCA-3'	For amplifying canine *RECK* cDNA 7
mF8	5'-TGTGCTGCCTACTCGGATCG-3'	For amplifying canine *RECK* cDNA 8
hR8	5'-TGTCCAGAGCAAGTGCAAGG-3'	For amplifying canine *RECK* cDNA 8
cF9	5'-TGCAAACCCATCATCCCACC-3'	For 3'RACE 1st PCR
cF10	5'-CTCTGCCCCTCCTACCTCCT-3'	For 3'RACE 2nd PCR
cR-RT	5'-TAGCCCCATCAAGGCCTGTA-3'	For reverse transcription of 5'end of canine *RECK* cDNA
cF-real	5'-TCCCTGAAGACCACACAGCT-3'	For real time PCR
RPS26F	5'-GCCATTAAGAAATTCGTCATTCG-3'	For real time PCR (internal standard)
RPS26R	5'-CGAGATCGATTCCTGACTAC-3'	For real time PCR (internal standard)
AUAP	5'-GGCCACGCGTCGACTCGTACTT(17)-3'	For reverse transcription of 3'RACE
UAP	5'-GGCCACGCGTCGACTCGTAC-3'	For 3'RACE 1st and 2nd PCR

'h' is based on the human *RECK* sequence (GenBank accession no. **NM_021111**), 'm' is mouse (**AB006960**) and 'c' is dog (**AB110699**). F: forward primer. R: reverse primer.

tions.

Quantitative RT-PCR analysis of canine RECK gene expression in normal tissues and canine tumor cells: Reverse-transcription was performed using total RNA samples isolated from various tissues (brain, heart, lung, liver, spleen, small intestine, pancreas, kidney, skeletal muscle, and testis) of three adult healthy male beagles and five canine tumor cell lines: osteosarcoma [11], high metastatic osteosarcoma [3], transitional cell carcinoma, mammary gland tumor (our unpublished cell lines), and mast cell tumor [9], and *RECK* expression was analyzed by quantitative RT-PCR method.

All PCR reactions were performed using a detection kit (Lightcycler-FirstStart DNA Master SYBR Green kit, Roche Molecular Biochemicals, Mannheim, Germany) [22]. For amplifying the *RECK* mRNA, cF-real and cR5 primers (Table 1) were used. To ensure the fidelity of mRNA extraction and reverse-transcription and to normalize the samples, all the samples were subjected to PCR amplification with oligonucleotide primers specific for the constitutively expressed gene *Ribosomal protein S26* (Table 1). The cycling conditions were as follows: initial denaturation at 95°C for 30 sec, followed by 35 cycles at 95°C touchdown (0 sec), 60°C for 5 sec, and 72°C for 10 sec. The identity of the PCR product was confirmed by electrophoresis.

Immunohistochemical analysis: Frozen tissue sections (6 μm) of acetone fixed testis and lung from a normal beagle dog were prepared as previously described [8]. Briefly, after pretreatment with normal rabbit serum, sections were incubated overnight at 4°C with polyclonal anti-mouse RECK antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) diluted (1:40) in PBS. The antigen-antibody reactions were visualized using biotin-labeled rabbit anti-goat IgG, peroxidase-streptavidin complex, and diaminobenzidine. Sections were counterstained with methyl green. A normal mouse neonate was used for the positive control, and the primary antibody was omitted for the negative control.

Transfection of canine RECK gene on tumor cells: Canine transitional cell carcinoma (TCC) cells were transfected with an expression plasmid vector (pFLAG-CMV™-2 Expression Vector, Sigma Chemical Co., St. Louis, MO, U.S.A.) carrying the canine *RECK* gene using the transfection reagent (FuGENE 6, Roche Molecular Biochemicals, Hague Road, IN, U.S.A.). A no-insert vector was used as a control. Transfection reagent/plasmid DNA complex formation was carried out according to the protocol supplied by the manufacturers. In brief, the transfection reagent (3 μl) and plasmid DNA (1 μg) were mixed in serum-free medium (97 μl) (Opti-MEM 1 Reduced-Serum Medium, Invitrogen) and incubated at room temperature for 45 min. Before 24 hr of transfection, TCC cells were seeded in RPMI-1640 (Invitrogen, Gland Island, NY, U.S.A.) with 10% FBS, in a 6-well plate. Then, the plasmid containing reagent was added into the newly changed medium with the TCC cells. Transfected cells were further incubated in 5% CO_2 at 37°C. Forty-eight hours after transfection, transduced cells were

selected for 2 to 3 weeks in RPMI-1640 medium containing 200–600 mg/m*l* G418. Messenger RNA expression of *RECK* gene was confirmed by the quantitative RT-PCR method.

Cell growth assay: Cell growth rates of the TCC cells, transfected and not transfected with the canine *RECK* gene, were analyzed by microtitration (MTT) assay [18]. Briefly, TCC cells (5×10^3 cells/well; 200 μl/well) suspended in RPMI-1640 with 10% FBS were placed in a flat bottom 96-well micotiter plates (IWAKI Co., Tokyo, Japan). After 1–4 days of incubation in 5% CO_2 at 37°C, 10 μl MTT solution (5 mg/m*l*) was added and cells were incubated for additional 3 hr at 37°C. The solution was then removed 100 μl and extraction buffer (20% SDS in 50% N,N-dimethylformamide) was added (100 μl/well) to dissolve the formazan pigment. After shaking for a few minutes, the plates were read on a multi-well scanning spectrophotometer at a wavelength of 570 nm.

Invasion and chemotaxis assays: Invasion and chemotaxis of *RECK* transfected TCC cells were assayed using Transwell (Corning, New York, NY, U.S.A.). Transwell has upper and lower compartments separated by polycarbonate membranes (pore size 8.0 μm). Cell invasion through the reconstituted basement membrane Matrigel (BD Biosciences, Bedford, MA, U.S.A.) was assayed by a method described previously [1]. Briefly, polycarbonate membranes of the upper compartment of Transwell culture chambers were coated with 0.1 mg/m*l* Matrigel (BD Biosciences, Bedford, M.A., U.S.A.). 2×10^5 cells suspended in RPMI1640 with 10% FBS were placed in the upper compartment of a Transwell chamber, and the lower compartment was filled with RPMI1640 containing 10 μg/m*l* fibronectin. Cells were incubated for 24 hr at 37°C under 5% CO_2. Then, the upper chamber was removed and the penetrated cells were fixed in 70% ethanol. The number of penetrated cells was counted after staining with Giemsa solution. Chemotaxis assay was performed under similar conditions without coating the upper chamber with Matrigel.

Statistical analysis: For the comparison of the canine *RECK* expression levels in tumor cells, analysis of variance followed by Scheffe's multiple comparison, as a post-hoc test, was used. For the comparison of cell growth, chemotaxis, and invasion in canine *RECK* gene transfected tumor cells and control, the Student's *t*-test was used. Differences were considered significant ($p<0.05$).

RESULTS

Determination of the cDNA sequence of canine RECK: The cDNA sequence and the deduced amino acid sequence of canine *RECK* were determined. We cloned 3,123 bases of canine *RECK* cDNA, and the open reading frame was suggested to be 2,913 bases in length (registered in GenBank; accession no. AB110699). The deduced amino acid sequence was 971 residues in length. Interstrain comparison of the nucleotide and amino acid sequences of canine *RECK* and previously reported mammalian *RECK* was done. Canine *RECK* cDNA showed high degree of homology with its human (92.5%) and mouse (87.1%) counterparts, and the predicted amino acid sequence of the protein showed 95.5% and 91.9% homology with human and mouse *RECK*, respectively (Fig. 1).

Expression of canine RECK mRNA in various tissues and tumor cells: The expression of canine *RECK* gene in brain, heart, liver, spleen, small intestine, pancreas, kidney, skeletal muscle, lung, and testis were examined by quantitative RT-PCR method. Variable levels of *RECK* expression were detected in various tissues (Fig. 2A). The highest expression level was detected in the lung and testis. As compared to normal tissue, a notably low canine *RECK* mRNA expression was detected in tumor cells. Among tumor cell lines, POS (osteosarcoma) showed significantly higher *RECK* expression (Fig. 2B).

Immunocytochemical localization of RECK in normal canine lung and testis: Immunohistochemical staining was performed in the lung and testis. The pseudostratified epithelium cells and the smooth muscle cells of the lung tissue showed strong immunoreactivity to the RECK antibodies (Fig. 3A). The head of the sperm cells in the testis also showed a strong immunopositive reaction (Fig. 3B). A strong reaction was observed in the positive control, especially in the vascular smooth muscle cells. No reaction was observed in the negative control.

Effects of transfected canine RECK gene on tumor growth, chemotaxis, and invasion: Canine *RECK* gene transfected into TCC cells did not show any significant effects on cell growth (Fig. 4A). We also could not detect any differences in chemotactic activity (Fig. 4B). In contrast, the matrix invasion activity was found to be significantly suppressed in canine *RECK* transfected cells as compared to no-insert vector-transfected control cells (Fig. 4C). Expression level of *RECK* mRNA was 24,907 times higher than the control cells.

DISCUSSION

RECK has been cloned in mouse and humans but not in dogs. In this study, we generated cDNA for canine *RECK* by the RT-PCR procedure. The cloned canine *RECK* displayed a strong amino acid identity with the human and mouse *RECK*. Canine *RECK* consists of 971 amino acids and was correlated to the human and mouse *RECK* in length [21]. Structural organization was investigated, and it was found that the NH_2-terminal one-third of the protein contains five repeats of a putative cysteine knot motif (6-Cys repeat: C_2-$X_{7\text{-}8}$-C-X_3-C-$X_{12\text{-}22}$-C-$X_{9\text{-}12}$-C; residues 37–84, 104–141, 151–197, 216–263, and 292–338) and five potential glycosylation sites (asparagines at positions 39, 86, 200, 297, and 352). The middle portion of the protein contains three serine-protease inhibitor-like domains, and the first domain (residues 635–654) completely matches the Kazal motif (C-X_7-C-X_6-Y-X_3-C-$X_{2,3}$-C). These structures are the features of *RECK* protein, and it is highly conserved in

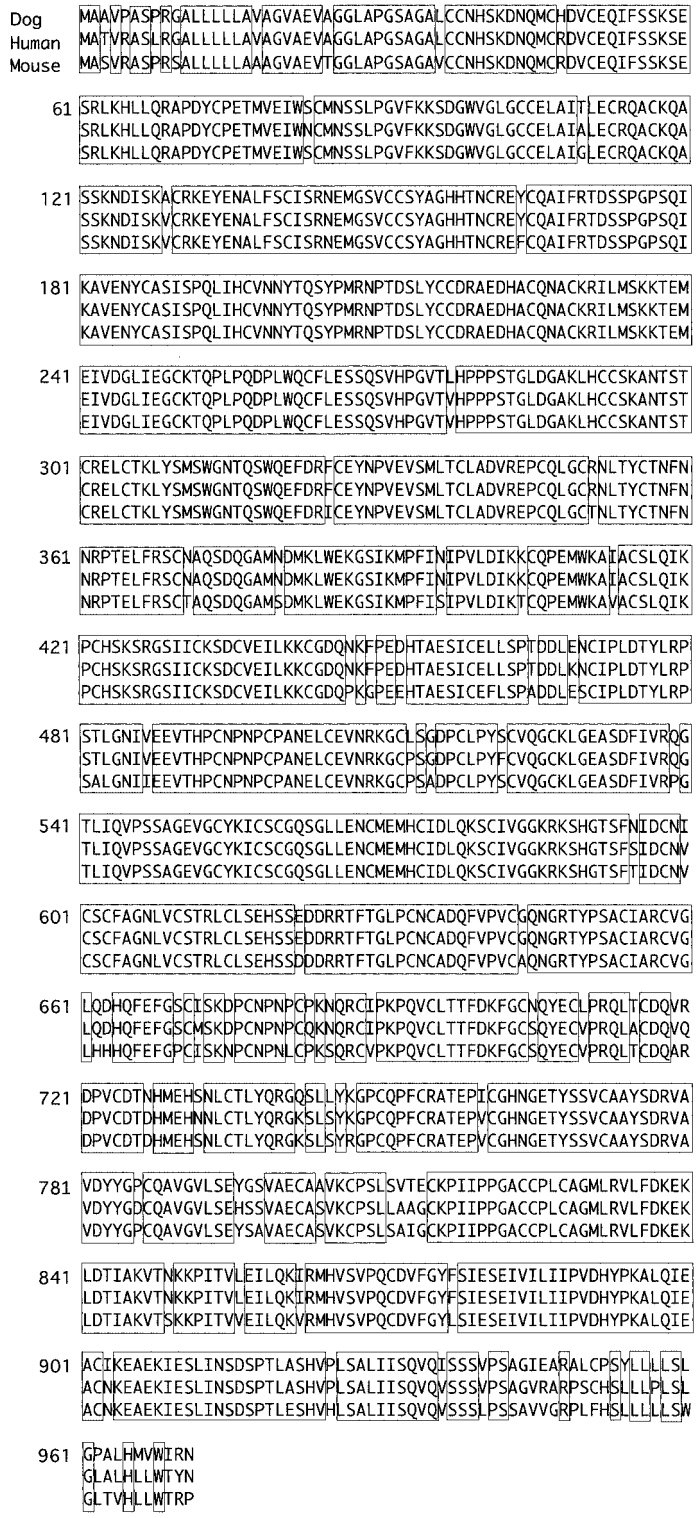

Fig. 1. Comparison of amino acid sequences of canine with the mouse and human reversion-inducing cysteine-rich protein with Kazal motifs (*RECK*). Identical amino acids are boxed.

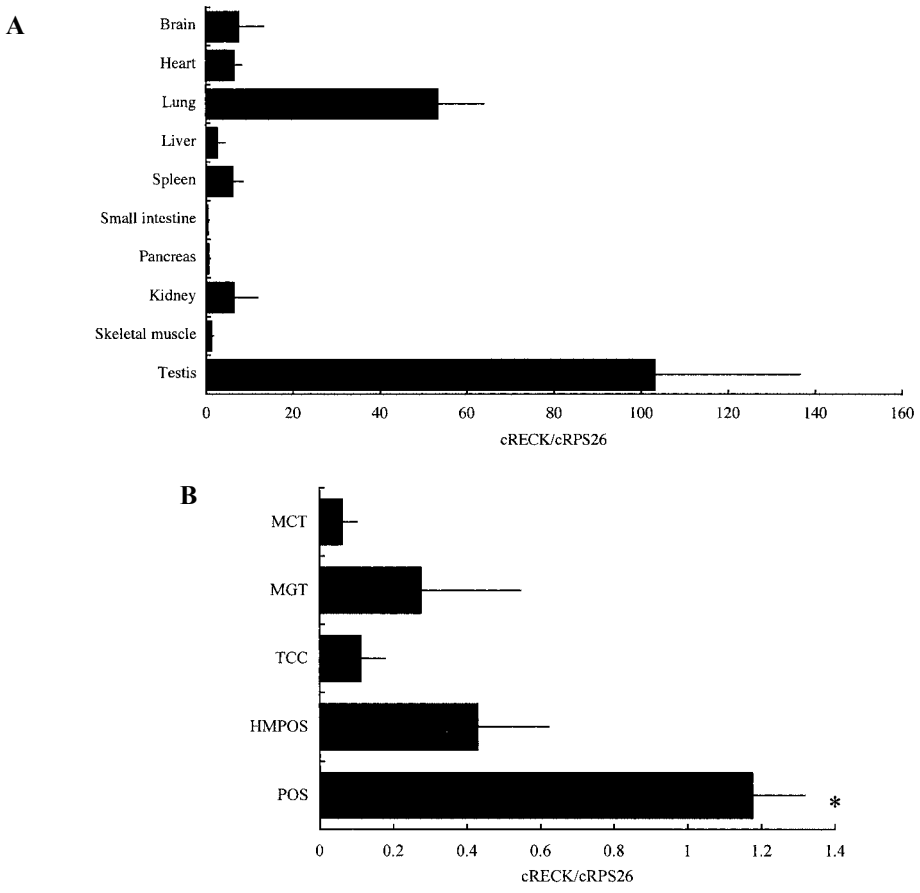

Fig. 2A. Normal tissue distribution of canine *RECK* mRNA. Lung and testis showed a higher expression of *RECK* mRNA as compared to other tissues. Each bar shows mean value of three normal tissues and error bars show standard deviation.

Fig. 2B. Tumor cell expression of canine *RECK* mRNA. MCT = Mast cell tumor, MGT = Mammary gland tumor, TCC = Transitional cell carcinoma, HMPOS = High metastatic osteosarcoma, and POS = osteosarcoma. Canine *RECK* expression in POS was significantly higher in POS in comparision with the other tumor cell lines. ($n = 3$, $p<0.05$)

human, mouse, and dogs at the amino acid level, suggesting that *RECK* has a biologically essential function beyond species.

The tissue distribution of *RECK* mRNA in dogs was also determined. *RECK* was highly expressed, especially in the lung and testis. Human *RECK* mRNA tissue distribution analyzed by Northern blotting also showed higher expression in the lung and testis [21]. The results from quantitative RT-PCR were confirmed by detecting a strong immunoreactivity in lung and testis by an immunohistochemical method. *RECK* expression was not detected by Northern blot in rodent and human malignant tumor cell lines of neuroblastoma, fibrosarcoma, malignant melanoma, transitional cell carcinoma, colon carcinoma, mammary grand tumor, retinoblastoma, leukemia, lymphoma, and hepatocellular carcinoma cells [21]. In the present study, low expression level of *RECK* mRNA was detected in various canine tumor cell lines by real-time quantitative PCR, which is much more sensitive than Northern blotting.

RECK expression was not investigated in rodent and human osteosarcoma cell lines [21]. In this study, we investigated the expression of *RECK* mRNA in osteosarcoma (POS), and it was detected to have a significantly higher expression as compared to the other tumor cell lines. In highly metastatic osteosarcoma (HMPOS), it was detected that *RECK* mRNA had a significantly lower expression as compared to the original osteosarcoma (POS). In a mouse osteosarcoma cell line, MMP-2 activity was higher in the high metastatic cell line than in the original cell line [2], suggesting that this could be the reason for the highly invasive and metastatic potential of the tumor.

It was reported that MMP activity in spontaneously developed canine tumors was higher than in unaffected stromal tissues, thus demonstrating that canine MMP-2 and MMP-9 activity might be involved in the pathogenesis of tumor metastasis [13, 14]. Therefore, RECK in canine

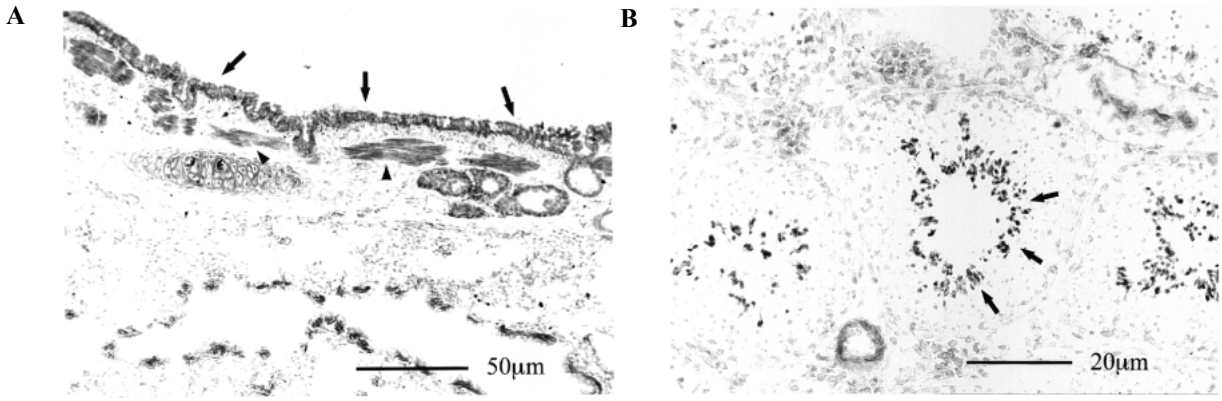

Fig. 3. Sections of normal canine tissue immunostained for *RECK*. (A) Lung. The bronchi chondrocytes (arrows) and the smooth muscle cells (arrow heads) showed immunopositive reactions. (B) Testis. Sperm (allows) showed strong immunopositive reactions.

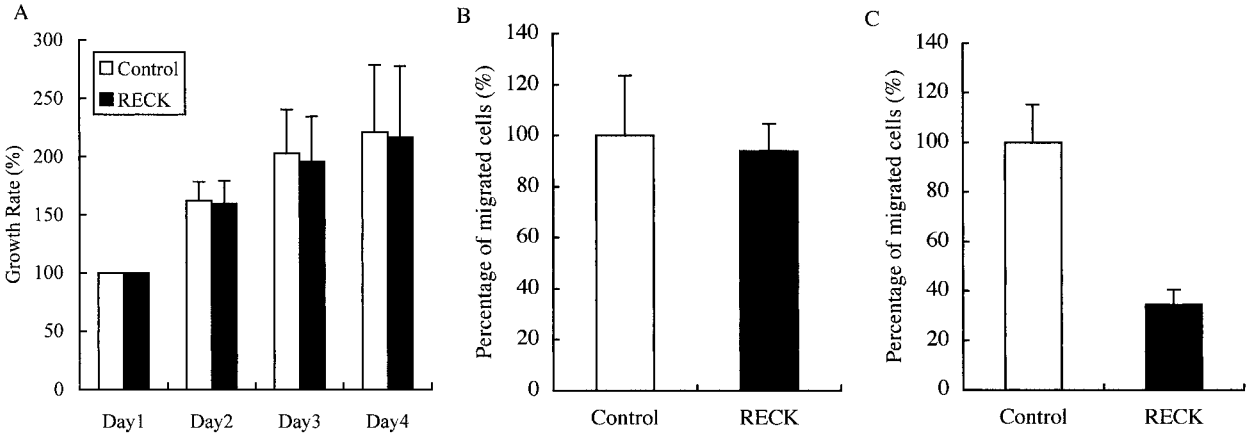

Fig. 4. Effects of transfected canine *RECK* gene on tumor growth (A: n=3), chemotaxis (B: n=6), and invasion (C: n=6). Cell growth and chemotaxis were not significantly influenced by transfection of canine *RECK*. Transfected canine *RECK* gene significantly suppressed tumor invasion.

tumor may be a good prognosis factor and malignancy marker as in the case of human beings [7, 19]. Naturally occurring canine tumors are believed to be a useful model of human cancer [5, 15]. The benefit of using a canine tumor model is that the biological behavior of the tumor is very similar to that of human tumors, but a shorter follow-up period is needed when investigating the survival time.

On the immunohistochemical examination of the lung and testis, strong immunoreactivity was detected in the pseudostratified epithelium cells and the head of the sperm, respectively. It might suggest that RECK has some potential to inhibit not only MMP-2, MMP-9, and MT1-MMP activity but also some other enzymes, such as hyaluronidase, esterase, acrosin, and neuraminidase. Further studies are required to identify other biological functions of *RECK* protein.

We checked the efficacy of transfected canine *RECK* gene on cell growth, chemotaxis, and invasion. Only tumor cell invasion was significantly suppressed. This result was closely related to the influence of RECK, which inhibits MMP-2 and MMP-9 activity. Since Matrigel is an extracellular matrix secreted by the Engelbreth-Holm-Swarm mouse sarcoma, in which the major components are laminin, collagen type IV, and other matrices [12], *RECK* transfected TCC cells could not degrade the collagen type IV and penetrate the membrane because they inhibited the MMP-2 and MMP-9 activity. This suggests that *RECK* down-regulation has an important relationship with the malignancy of the tumor cells.

In conclusion, our results suggested that RECK might be correlated to tumor malignancy in dogs as well as in other mammalians. Finally, we took one more step in understanding canine tumor pathology, which is closely related to human tumors.

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