Lentivirus-mediated reversion-inducing cysteine-rich protein with Kazal motifs gene transfer suppresses pancreatic cancer invasion in vitro

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ABSTRACT

الأهداف: دراسة الوظائف الحيوية للانعكاس المنتج للبروتين الغني بالسيسئين مع عنصر (RECK) المنتشر بكثرة في خطوط خلايا سرطان البنكرياس.

الطريقة: أجريت الدراسة في قسم الجراحة العامة التابع لمستشفى جامعة سوتشو، سوتشو، جيانغسو، الصين خلال الفترة من يناير إلى أغسطس 2009م. أجريت الدراسة لإنشاء الفيروسات البطيئة المأشوبة الحاملة لجين (RECK) واستخدمت لإفساد خلايا السرطان وفحص وظائف (RECK) الحيوية في سرطان البنكرياس في المختبر. تم قياس ظهور (RECK) بسلسلة التفاعل المبلمر، ولطخة وسيترن. كما تم فحص تكاثر الخلايا وموتها بواسطة ديموثليزل وقياس الانسياب الخلوي. كما اكتشفت قدرة الانتشار بغرف ترانزول. وتم قياس نشاط ظهور بروتينز2 و بروتينز9 بتفاعل السلسلة المبلمرة، ولطخة ويسترن، ومقياس هلامية الإنزيم.

النتائج: أنشئنا بنجاح جين (LV-RECK)، وأثبتت قدرة على الانتشار في خلايا البنكرياس. أن انتشار جين (RECK) قلل من قدرة الانتشار في خلايا البنكرياس في المختبر والتأثير على تكاثر الخلايا وموتها. قلل انتشار جين (RECK) على الإفراز ونشاط (MMP2)، وإفراز (MMP9) بدون التأثير على حامل الحمض النووي وظهور بروتين (MMP2) و (MMP9).

خاتمة: سعى جين (RECK) على الضغط والتأثير على قدرة الانتشار في خطوط خلايا البنكرياس السرطانية ويعد عامل علاج فعال لسرطان البنكرياس.

Objectives: To investigate the biological functions of reversion-inducing cysteine-rich protein with Kazal motifs (RECK) over-expression in pancreatic cancer cell line Panc-1.

Methods: This study was carried out in the Department of General Surgery, First Affiliated Hospital of Soochow University, Suzhou, Jiangsu, China from January to September 2009. The design of the study was to construct a recombinant lentivirus carrying the RECK gene (LV-RECK) to be used to infect Panc-1 cells, and investigate the biological functions of RECK in pancreatic cancer in vitro. The RECK expression was measured by real-time polymerase chain reaction (PCR) and Western blotting. Cell proliferation and apoptosis were examined by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry. The ability of invasion was detected using Transwell chambers. The expression and activity of matrix metalloproteinase (MMP)-2 and matrix metalloproteinase-9 (MMP-9) was measured by real-time PCR, Western blotting, and gelatin zymography.

Results: We successfully constructed LV-RECK and proved it can over-express the RECK gene in Panc-1 cells. The RECK over-expression potently suppressed the invasion ability of Panc-1 cells in vitro without affecting cell proliferation and apoptosis. The RECK over-expression potently inhibited the secretion and activity of MMP-2, and the secretion of MMP-9 without affecting the messenger ribonucleic acid, and protein expression of MMP-2 and MMP-9.

Conclusion: The RECK gene exerts repressive effects on the invasion ability of the pancreatic cancer cell line Panc-1, and it may be a novel therapeutic target for pancreatic cancer.

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Pancreatic cancer remains the fourth leading cause of cancer mortality in the United States.[1] Due to the aggressive natural history of this disease, most patients with pancreatic cancer have been found with local invasion, or distant metastasis at the time of diagnosis, and less than 20% of patients are candidates for surgery with curative intent.[2] To improve the prognosis, understanding the molecular mechanisms of invasion and metastasis in pancreatic cancer is needed. The reversion-inducing cysteine-rich protein with Kazal motifs (RECK) gene was isolated as a transformation suppressor gene by screening a fibroblast expression library for complementary DNAs that induced nontransformed flat morphology when expressed in v-Ki-ras-transformed NIH3T3 (a mouse embryonic fibroblast cell line) cells.[3] The RECK gene encodes a glycosylphosphatidylinositol-anchored glycoprotein of approximately 110 kiloDaltons (kDa). The RECK messenger ribonucleic acid (mRNA) is expressed ubiquitously in normal human organs and non-neoplastic cell lines, but its expression is strongly suppressed in a number of tumor-derived cell lines, or in cells expressing several active oncogenes.[3-6] Additionally, the decrease in RECK expression is reported to correlate with hypermethylation and deacetylation of the promoter region.[4,7-9] Over-expression of RECK in tumor cells decreases the amount of active matrix metallopeptidase (MMP)-2 and MMP-9 in conditioned medium, and suppresses tumor metastatic activity in vitro,[3,10-13] and in vivo.[14] Clinical investigations also demonstrated that patients with high RECK protein in tumor tissues tended to show better survival, and such tumors were less invasive.[15-20] These data suggest that RECK is a novel suppressor gene for metastasis, and is likely to be a good target for cancer therapy. In this study, we used a recombinant RECK lentivirus to over-express the RECK gene in Panc-1 cells, with the aim of evaluating its role in pancreatic cancer cell invasion in vitro.

Methods. This study was carried out in the Department of General Surgery, the First Affiliated Hospital of Soochow University, Suzhou, Jiangsu, China from January to September 2009. The study protocol was approved by the Medical Ethical Committee of our institution. The coding domain sequence of human RECK gene (GenBank, NM_021111) was amplified by polymerase chain reaction (PCR) from plasmid pINCY (GeneChem Company, Shanghai, China). The amplified product was inserted into the lentiviral expression plasmid pGC-FU (lentiviral shuttle plasmid containing enhanced green fluorescent protein [EGFP]) and ligation. Recombinant lentiviruses carrying RECK gene (LV-RECK) and control virus (LV-NC) were produced by 293T cells following the co-transfection of the lentivirus expression plasmid, and packaging plasmids. Human pancreatic cancer cell line Panc-1 was maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The viral supernatant was added into Panc-1 cells at a multiplicity of infection (MOI) of 10. The cells infected with LV-NC, or LV-RECK were named as Panc-1/NC or Panc-1/RECK. After infection with lentiviral vector, the expression of target gene usually started at day 2, reached a peak at day 5. So we started further experiments at 96 hrs after infection. Quantitative real-time PCR assays were carried out. Human actin was used as internal control. The PCR conditions consisted of one cycle at 95°C for 15 seconds (s) followed by 45 cycles at 95°C for 5 s, and at 60°C for 30 s. The primer sequences for the genes and expected product sizes were as follows: 5'-ACTAATCCAGGTGCCATCATC-3' sense, 5'-TTCCTCCAACAATACAAGACTTC-3' antisense for RECK (134 bp), 5'-ACCACAGCCAACTACGATG-3' sense, 5'-TGCCAAGGTCAATGTCAGG-3' antisense for MMP-2 (235 bp), 5'-GCACCACCACAACATCAC-3' sense, 5'-ACCACAACTCGTCATCGTC-3' antisense for MMP-9 (284 bp), 5'-GGCGGCACCACCATGTACCCT-3' (sense), 5'-AGGGGCCGGACTCGTCATACT-3' antisense for actin 202 bp. Real-time PCR results were analyzed using the $2^{-\Delta\Delta Ct}$ method. Ninety-six hrs after infection, cells were washed with phosphate buffer solution (PBS), suspended in lysis buffer, and lysed on ice. Supernatant of the lysate with equal amounts of total protein was then separated on 10% sodium dodecyl sulfate (SDS) polyacrylamide gels with a 30% polyacrylamide gel, and transferred onto polyvinylidene diflouride membrane. Membranes were blocked with 5% nonfat dry milk/tris-buffered saline (TBS)/0.1% Tween-20 incubated with anti-RECK, anti-MMP-2, anti-MMP-9, anti-glyceraldehyde-3-phosphate dehydrogenase, and anti-ß-actin antibody (all from Santa Cruz Biotechnology, CA, USA), washed 3 times with TBS/0.1% Tween-20, then incubated with a goat anti-mouse immunoglobulin G (Santa Cruz Biotechnology, CA, USA). Cell proliferation was assessed using the 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in 96-well culture plates in culture medium at an optimal density ($5x10^3$ cells per well) in triplicate wells for the Panc-1/RECK, Panc-1/NC, and Panc-l cells groups. After 0, 24, 48, 72, 96, and 120 hrs of incubation, cells were stained with 20 µL MTT (5 mg/mL) at 37°C for 4 hrs, and subsequently made soluble in 150 mL of dimethyl sulfoxide. Absorbance was measured at 570 nm. Apoptosis was analyzed by flow cytometry with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) double labeling.

Briefly, cells were collected, washed with cold PBS, and then suspended in annexin V-binding buffer. The FITC-conjugated annexin V (5 µL) and PI (10 µL) were added to the cells, and incubated for 15 minutes. The samples were analyzed by flow cytometry within one hour. The total number of apoptotic cells was determined by calculation of annexin V^+/PI^- cells together with annexin V^+/PI^+ cells.

Cell invasion assay was performed using Transwell chambers. Briefly, Panc-1/RECK, Panc-1/NC and Panc-l cells ($1x10^5$) suspended in serum-free media containing 0.1% bovine serum albumin were placed in the upper chambers, and the lower chambers were filled with DMEM containing 10% FBS. After 24 hrs incubation at 37°C, the non-invading cells were removed from the upper surface of the filters by wiping with a cotton swab. The cells on the lower surface of the filters were fixed with cold methanol, stained with 0.2% crystal violet, and counted under a light microscope. Furthermore, Panc-1/RECK, Panc-1/NC and Panc-l cells ($1x10^5$) in logarithmic growth phase were cultured using serum-free DMEM for 24 hrs. The conditioned medium was harvested and analyzed for zymography. Equal volumes of the samples were separated by electrophoresis through 10% SDS-polyacrylamide gels containing 0.1% (w/v) gelatin. After the electrophoresis, the gels were washed with a solution containing 2.5% Triton X-100 (BDH Ltd, Dorset, UK) to remove SDS, rinsed with 50 mmol/L Tris/hydrochloric acid, and then incubated in reaction buffer. The gels were stained with 0.05% Coomassie Brilliant Blue. After destaining with 30% methanol and 10% acetic acid, the gelatinolytic activity was visualized as clear white bands against a blue background. Each experiment was performed at least 3 times and found to be reproducible.

The statistical significance of differences between the groups was determined by applying one-way analysis of variance (ANOVA), followed by Tukey's Honestly Significant Difference (HSD). A $p<0.05$ was considered statistically significant. These analyses were performed using the Statistical Package for Social Sciences version 13.0 software (SPSS Inc, Chicago, IL, USA).

Results. We successfully constructed LV-RECK and Panc-1 cells infected with LV-RECK or LV-NC at high levels of efficiency. Moreover, there was almost no cytotoxicity in the LV-NC-infected Panc-1 cells at an MOI of 10. To determine the effect of LV-RECK on the expression of RECK, real-time PCR, and Western blotting were performed. As shown in **Figure 1a**, RECK mRNA expression was significantly increased in the Panc-1/RECK group compared with Panc-1 and Panc-1/NC groups ($p=0.000$), but there were no significant differences between Panc-1/NC and Panc-1 groups ($p>0.05$). Approximately 110 kDa protein band, RECK protein, was detected only in Panc-1/RECK cells, but not in the Panc-1/NC or Panc-1 cells (**Figure 1b**). To investigate the effect of RECK on Panc-1 cells growth, cell proliferation was assessed using the MTT assay. As a result we found that there were no differences in cell proliferation and viability between the 3 groups ($p>0.05$) (**Table 1**). The effect of RECK on Panc-1 cells apoptosis was evaluated with flow cytometry analysis. As shown in **Figure 2**, there were no significant differences in cell apoptosis between the 3 groups ($p>0.05$). To investigate

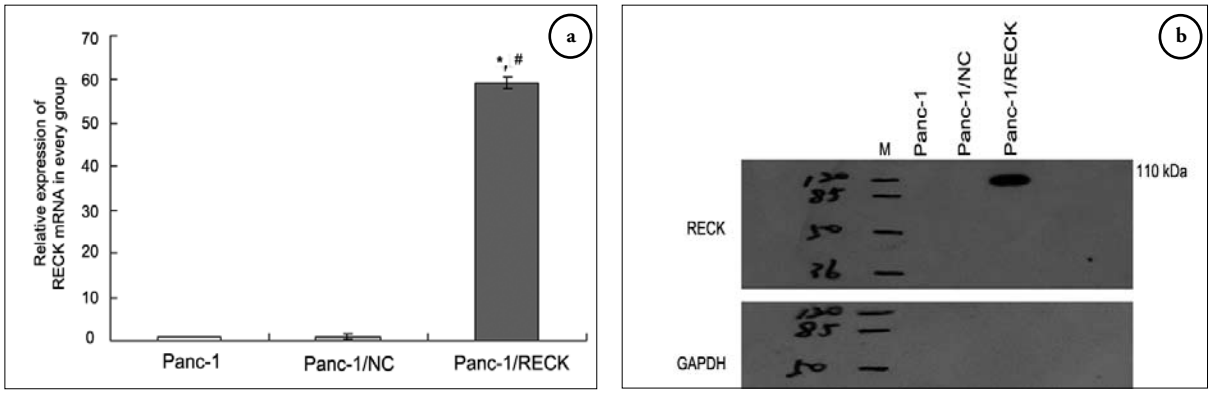

Figure 1 - Over-expression of reversion-inducing cysteine-rich protein with Kazal motifs (RECK) by recombinant lentiviruses carrying RECK gene (LV-RECK) in Panc-1 cells showing: **a**) messenger ribonucleic acid (mRNA) level of RECK detected by real-time polymerase chain reaction after Panc-1 cells were infected with LV-RECK and control virus (LV-NC). The LV-RECK significantly increased expression of RECK mRNA in Panc-1 cells, and **b**) Western blotting analysis showed that RECK protein expression was restored by LV-RECK in Panc-1 cells. $^*p=0.000$ versus Panc-1 group, $^\#p=0.000$ versus Panc-1/NC group ([Panc-1 cells infected with LV-NC]), GAPDH - anti-glyceraldehyde-3-phosphate dehydrogenase. M - marker

Table 1 - Influence of RECK over-expression on Panc-1 cell proliferation.

Groups	Absorbance in hours (mean ± SD)					
	0	24	48	72	96	120
Panc-1	0.218 ± 0.017	0.320 ± 0.018	0.420 ± 0.019	0.659 ± 0.021	0.862 ± 0.031	0.957 ± 0.037
Panc-1/NC	0.213 ± 0.013	0.296 ± 0.014	0.405 ± 0.007	0.638 ± 0.013	0.842 ± 0.022	0.938 ± 0.019
Panc-1/RECK	0.216 ± 0.019	0.291 ± 0.019	0.395 ± 0.019	0.627 ± 0.014	0.820 ± 0.019	0.926 ± 0.020

SD - standard deviation, RECK - reversion-inducing cysteine-rich protein with Kazal motifs, NC - control virus

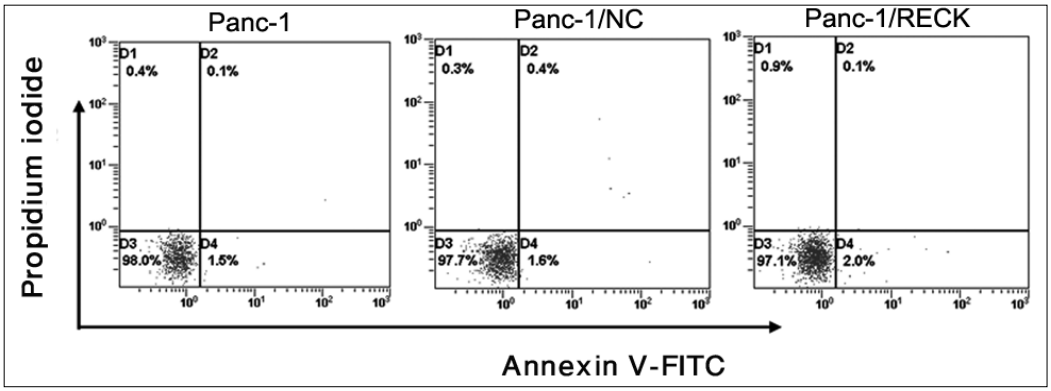

Figure 2 - Effect of lentiviruses-reversion-inducing cysteine-rich protein with Kazal motifs (RECK) on Panc-1 cells apoptosis. Quadrants D2-D4 represent necrotic/late apoptotic cells, viable cells, and early apoptotic cells. V-FITC - V-fluorescein isothiocyanate. Vertical arrow represents propidium iodide and horizontal arrow represents annexin V-FITC. Shaded areas stand for scatter plot. D - has no special meaning, but Dx represents different quadrant as mentioned above.

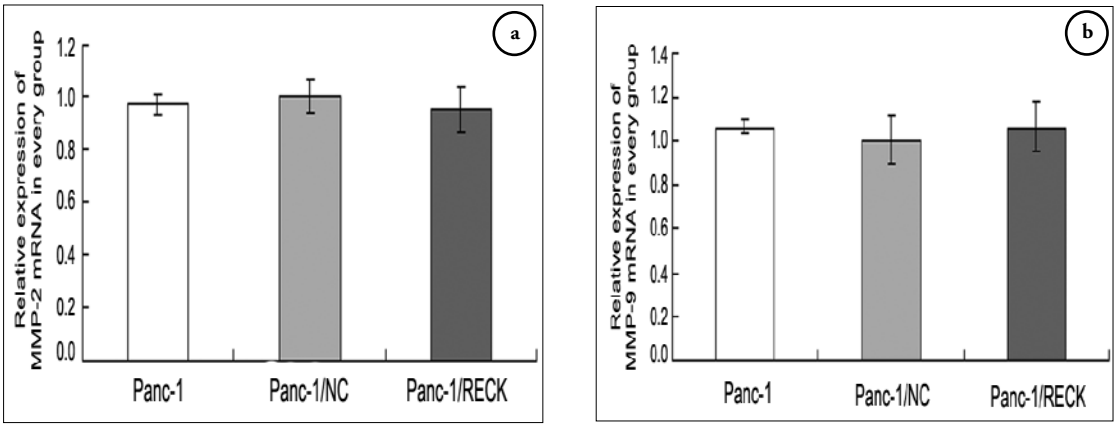

Figure 3 - Effect of lentiviruses (LV)-reversion-inducing cysteine-rich protein with Kazal motifs (RECK) on matrix metalloproteinase (MMP)-2, and MMP-9 messenger ribonucleic acid (mRNA) expression in Panc-1 cells. **a**) relative expression of MMP-2 mRNA in every group, **b**) relative expression of MMP-9 mRNA in every group.

the role of RECK in the invasiveness of pancreatic cancer cells the invasion ability of Panc-1 cells, before and after LV-RECK infection was evaluated using a Transwell chamber assay. As shown in **Table 2**, the number of penetrated cells in Panc-1/RECK group was significantly lower than in Panc-1 and Panc-1/NC groups (p=0.000), but there were no significant differences between Panc-1/NC and Panc-1 groups (p>0.05). The results indicate that infection of the RECK gene significantly reduced the invasion ability of Panc-1 cells. To elucidate the molecular mechanism involved in LV-RECK-mediated Panc-1 tumor cells invasion suppression, the expression

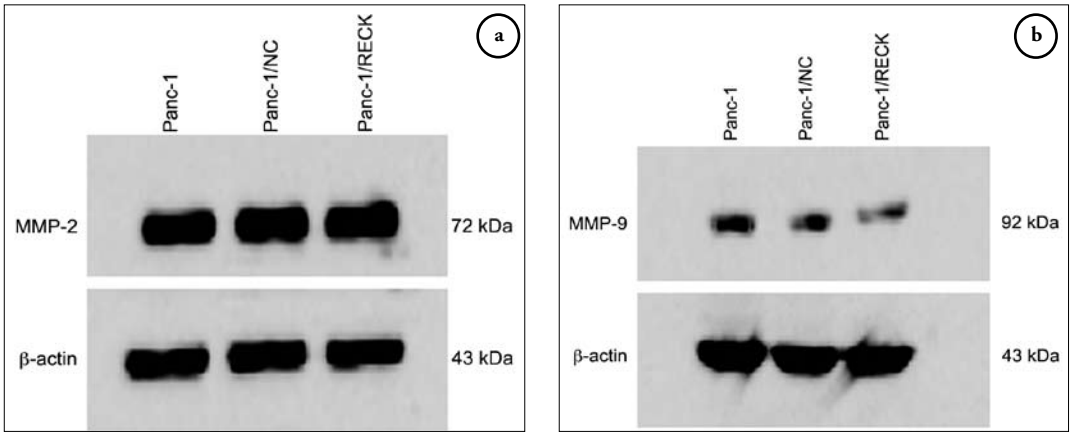

Figure 4 - Effect of lentiviruses (LV)-reversion-inducing cysteine-rich protein with Kazal motifs (RECK) on matrix metalloproteinase (MMP)-2, and MMP-9 messenger ribonucleic acid (mRNA) expression in Panc-1 cells, **a**) expression of MMP-2 protein in every group, **b**) expression of MMP-9 protein in every group.

Table 2 - Influence of RECK over-expression on Panc-1 cell invasion.

Groups	Invasive cell number mean ± SD
Panc-1	61.133 ± 4.392
Panc-1/NC	57.667 ± 1.332
Panc-1/RECK	19.933 ± 2.730*,†

RECK - reversion-inducing cysteine-rich protein with Kazal motifs, SD - standard deviation, *p=0.000 versus Panc-1 group, †p=0.000 versus Panc-1/control virus (NC) group.

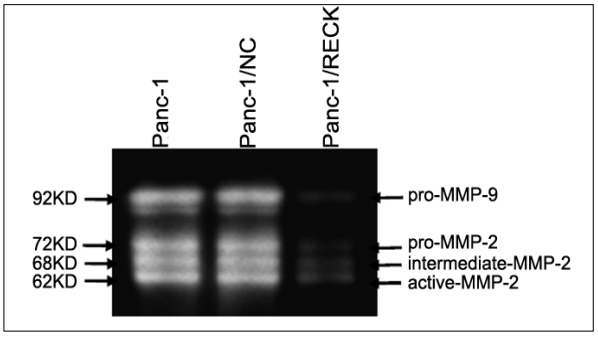

Figure 5 - Effect of lentiviruses (LV)-reversion-inducing cysteine-rich protein with Kazal motifs (RECK) on matrix metalloproteinase (MMP) -2, and MMP-9 activities in Panc-1 cells. KD - kiloDalton

of the invasion-related genes, MMP-2 and MMP-9 in LV-RECK-infected Panc-1 cells was examined by real-time PCR and Western blotting. As shown in **Figures 3** & 4, over-expression of RECK did not affect the mRNA and protein expression of MMP-2 and MMP-9 in Panc-1 cells (p>0.05). To investigate whether restoring the expression of RECK might lead to the inhibition of MMP activity, conditioned medium of Panc-1 cells after LV-RECK infection were analyzed by gelatin zymography. As shown in **Figure 5**, gelatinolytic activities of pro-, intermediate- and active-MMP-2, and pro-MMP-9 examined by zymography were significantly diminished in Panc-1/RECK cells compared with Panc-1 and Panc-1/NC cells, but there were no significant differences between Panc-1/NC and Panc-1 groups (p>0.05). It is noteworthy that the total amounts of MMP-2 (including pro-, intermediate, and active form) secreted by Panc-1/RECK cells were also reduced.

Discussion. Previous experimental studies have revealed that RECK is able to inhibit tumor progression through inhibiting tumor angiogenesis, invasion, and metastasis.[3,8,10,21,22] In addition, clinical studies indicated that RECK is downregulated in many types of solid tumors, and the extent of downregulation often correlates with poor prognosis.[23] However, the biological functions of RECK in pancreatic cancer is largely lacking. In this study, we succeeded in constructing recombinant RECK lentivirus, which could infect Panc-1 cells efficiently. The result of real-time PCR and Western blotting demonstrated that the recombinant lentivirus vector successfully over-expressed the mRNA and protein of RECK in Panc-1 cells, which is critical for further functional studies in vitro.

On the basis of over-expression of RECK mRNA and protein, we investigated the effects of RECK gene on the proliferation, apoptosis, and invasion of human highly metastatic pancreatic cancer cell line Panc-1. We found that infection of the RECK gene significantly inhibited the invasion potential of Panc-1 cells in vitro, without affecting cell proliferation and apoptosis. These

results showed that the observed decrease in invasion was not the result of reduced cell proliferation and increased apoptosis. Our findings are in line with the study of Takahashi et al,[3] in which restored expression of RECK in tumor-derived cell lines does not alter cell viability, growth, motility, or chemotactic activity of malignant cells in vitro or in vivo, but does attenuate in vitro invasion capacity, and in vivo metastasis formation in experimental metastasis assays.

Cancer metastasis is a complex multistep process, in which invasion of the extracellular matrix and basement membrane by tumor cells is a critical step. The MMPs play critical roles in this step by degrading components of the basement membranes and extracellular matrix. Among the more than 20 MMPs that have been identified,[24] MMP-2 and MMP-9 specifically degrade type IV collagen, the major structural component of basement membrane. Previous studies indicated knockdown of MMP-2 or MMP-9 by ribonucleic acid interference significantly inhibits the invasive potential of pancreatic cancer cells in vitro.[25,26] Earlier studies by Takahashi et al[3] and Oh et al[10] reported that RECK suppressed tumor cells invasion by inhibiting the secretion and activity of MMP-2, MMP-9, and membrane-type 1 MMP (MT1-MMP/MMP-14). Takagi et al[27] reported that RECK negatively regulates MMP-9 transcription. In this study, we found that over-expression of RECK did not affect the mRNA and protein expression of MMP-2 and MMP-9 in Panc-1 cells. However, gelatin zymography showed that the levels of pro-, intermediate- and active-MMP-2 and pro-MMP-9 in the supernatant were significantly decreased after forced expression of RECK in Panc-1 cells (note that during this assay, the total amount of MMP-2 secreted by Panc-1/RECK cells were also reduced). Our findings collectively suggest that RECK regulates MMP-2 and MMP-9 posttranslationally, such as inhibiting the activation cascade of MMP-2 and the secretion of MMP-9 and MMP-2, which in turn suppress the invasion capacity of Panc-1 cells.

We considered that more pancreatic cancer cell lines should be included in this study besides Panc-1 to confirm the availability of suppression of invasion mediated by RECK on pancreatic cancer cells. Furthermore, animal experiments should also be performed to further confirm the inhibitory effects of metastasis mediated by RECK on pancreatic cancer in vivo. Therefore, the anticancer effect induced by RECK require further investigations, and we will engage in this field further. In conclusion, our findings indicate that RECK can potently suppress pancreatic cancer cell line Panc-1 invasion, and it may be a promising target for the treatment of pancreatic cancer.

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Related topics

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