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REG4 contributes to the invasiveness of pancreatic cancer by upregulating *MMP-7* and *MMP-9*

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Recent studies have shown that overexpression of regenerating gene family member 4 (*REG4*) is associated with the initiation and progression of pancreatic cancer. In our study, we explored the role of *REG4* in the invasion of pancreatic cancer. Real-time PCR and Western blot analysis were used to determine *REG4* expression in pancreatic cancer cell lines. An MTT assay was carried out to test the effect of *REG4* on the growth of pancreatic cancer cells. The involvement of *REG4* in cancer cell invasion was examined by Transwell invasion assay. Two MMPs, *MMP-7* and *MMP-9*, were identified from a pool of candidate genes as being related to *REG4*-induced cell invasion by PCR and Western blotting. Immunohistochemistry was used to confirm the correlation between *REG4* and the two MMPs. High expression of *REG4* was found in BXPC-3 cells and its culture media. But in PANC-1 and ASPC-1 cell lines, *REG4* expression levels were very low, and no detectable protein was found in the culture medium. The MTT and Transwell invasion assays showed that recombinant REG4 protein and BXPC-3 conditioned media significantly promoted the proliferation and invasiveness of pancreatic cancer cells. It was also shown that MMP-7 and MMP-9 are upregulated by *REG4* induction using real-time PCR and Western blotting analysis. Immunohistochemical study further verified this result. In conclusion, *REG4* promotes not only growth but also *in vitro* invasiveness of pancreatic cancer cells by upregulating MMP-7 and MMP-9. (*Cancer Sci* 2012; 103: 2082–2091)

Pancreatic cancer remains one of the most difficult malignancies to diagnose and treat. It is estimated to be the fourth most common cancer in men and fifth in women worldwide. Despite the development of surgical resection, radiotherapy, and chemotherapy, the majority of patients are diagnosed when the disease has reached an advanced stage. The prognosis of pancreatic cancer remains miserable as the 5-year survival rate is still less than 5%.[1,2] The salient features of pancreatic cancer are extensive local invasion, early systemic dissemination, and poor prognosis. These characteristics are mainly attributed to the malignant behavior of pancreatic cancer cells which have highly invasive and metastatic potential. Therefore, the study of the molecular mechanisms behind the invasion and metastasis of pancreatic cancer is urgently needed.

Regenerating gene family member 4 (*REG4*) was originally identified by sequencing of a cDNA library derived from patients with inflammatory bowel disease. It is located on chromosome 1, encoding 158 amino acids, including a signal peptide of 22 amino acids and a conserved calcium-dependent carbohydrate-recognition domain.[3] Although *REG4* is expressed in various normal tissues, the expression levels are much lower than in cancerous tissues. REG4 was found to be markedly upregulated in pancreatic, colorectal, gastric, gall bladder, and prostate cancer tissues compared to paired normal mucosa.[4–9] Several studies have shown that serum REG4 level can serve as a diagnostic and prognostic marker in colorectal, gastric, and pancreatic cancer.[7,10,11] Our previous study on pancreatic cancer suggested that the serum REG4 level is higher in pancreatic cancer patients than in patients with benign pancreatic disease and healthy controls, and concurrent testing of REG4 and CA19-9 can increase the diagnostic sensitivity to 90.5%.[12] Studies on colorectal cancer reported that upregulation of REG4 is associated with tumor differentiation, stage, and lymph node metastasis and may act as a pro-invasive factor.[13,14] Cell treatment *in vitro* indicated that REG4 can regulate normal intestinal and colorectal cancer cell susceptibility to radiation-induced apoptosis by increasing the expression of anti-apoptotic genes *Bcl-2*, *Bcl-XL*, and *survivin*.[15] Hu *et al.*[16] reported that recombinant human REG4 protein (rREG4) can protect against arginine-induced necrosis of pancreatic acinar cells both *in vivo* and *in vitro* by upregulating *Bcl-2* and *Bcl-xL* expression and activating the epidermal growth factor receptor/protein kinase B (EGFR/Akt) pathway. In hormone-resistant prostate cancer, overexpression of *REG4* is associated with cancer progression and metastasis.[17] Our previous study on gastric cancer also revealed that *REG4* expression correlates with Lauren classification, differentiation, lymph node metastasis, distant metastasis, and TNM stage, and patients with high *REG4* expression had short survival time and poor outcome.[18] Other studies showed that stable expression of *REG4* significantly enhances peritoneal metastasis in gastric cancer, indicating that REG4 may serve as a novel marker for detecting peritoneal dissemination.[19,20] In summary, *REG4* appears to play an important role in cancer development by enhancing proliferation, inhibiting apoptosis, and promoting metastasis and peritoneal dissemination, but the detailed mechanism remains poorly understood.

In this study, rREG4, REG4 antibody, and REG4 siRNA were used to investigate the functions of *REG4* on pancreatic cancer cell proliferation, migration, and invasion with the help of MTT and Transwell assays. To complement this *in vitro* data, a pool of candidate genes were examined for their involvement in *REG4*-induced cell invasion using real-time RT-PCR and Western blot analysis. Immunohistochemical staining was also used to further validate the correlation between *REG4* and the expression levels of the two MMPs, MMP-7 and MMP-9.

Materials and Methods

Cell culture. Three pancreatic cancer cell lines with different levels of differentiation including PANC-1 (poorly differentiated), ASPC-1 (moderately to well differentiated), BXPC-3 (well differentiated), and mice NIH 3T3 fibroblast cells were

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They were cultured in DMEM (HyClone, Logan, UT, USA) supplemented with 10% FBS and antibiotics (100 U/mL streptomycin and 100 U/mL penicillin) and maintained at 37°C in 5% CO_2. Cells were passaged at 80% confluency using 1 mmol/L EDTA–0.025% trypsin for 3–5 min.

Clinical samples. This study enrolled 40 patients with pancreatic cancer who underwent pancreatectomy without preoperative radiotherapy or chemotherapy at Zhejiang Provincial Peoples' Hospital (Hangzhou, China) between 2009 to 2011. Specimens were fixed in formalin and embedded in paraffin. Before the study, written informed consent was obtained from all the patients and the project was approved by the ethics committee of Zhejiang Provincial People's Hospital.

Cell transfection. *REG4* siRNA (sc-61448; Santa Cruz Biotechnology, Santa Cruz, CA, USA) mixed with siRNA transfection reagent (sc-29528; Santa Cruz Biotechnology) was used for transfection according to the manufacturer's instructions. In 6-well plates, 2×10^5 BXPC-3 cells were transfected with *REG4* siRNA or control siRNA (sc-37007, Santa Cruz Biotechnology Inc., USA). After 24 h, cells were collected for subsequent experiments.

***REG4* conditioned media.** BXPC-3 cells transfected with *REG4* siRNA and the controls were seeded into 75-cm^2 cell culture bottles. When the cell growth reached 80% confluence, the culture medium was replaced by serum-free DMEM. After 24 h of incubation at 37°C with 5% CO_2, the supernatants were collected and stored at 4°C. The BXPC-3 conditioned media with *REG4* siRNA was named CM_{siREG4}, and the conditioned media with control siRNA was named CM.

A sample of the CM was then incubated with 1 μg/mL REG4 mAb (Mab1379; R&D Systems, Minneapolis, MN, USA) for 2 h, and was named CM_{Ab}.

Cell proliferation assay. Cells were seeded in 96-well plates at a density of 5×10^3 cells/well for PANC-1 and 1.0×10^4 cells/well for ASPC-1. After 24 h, cells were divided into eight groups. Group 1 was used as control, and groups 2–5 were treated with four different concentrations (0.25, 0.5, 1, 2 μg/mL) of rREG4 (1379-RG; R&D Systems). Group 6 was treated with CM, group 7 treated with CM_{siREG4}, and group 8 treated with CM_{Ab}. The MTT method[21] was used to detect the effect on cell proliferation at different time intervals (24, 48, and 72 h). The relative cell proliferation (%) was obtained by the following equation, where A represents absorbance:

$$\text{Relative proliferation rate}\,(\%) = \frac{(A_{570\text{nm}}) - (A_{630\text{nm}})\ \text{of study group}}{(A_{570\text{nm}} - A_{630\text{nm}})\ \text{of control group}} \times 100\%.$$

Transwell assay. Five groups of BXPC-3 cells were taken. Two groups were transfected with REG4 siRNA (3 μL and 6 μL), another was incubated with 1 μg/mL REG4 mAb, and two were used as the blank control and siRNA control. Then BXPC-3 cells were collected and suspended in serum-free DMEM at a density of 2.0×10^6 cells/mL. Five samples of PANC-1 cells were taken. One was suspended in serum-free DMEM containing 2 μg/mL rREG4, the second suspended in CM, the third suspended in $CM_{Ab,}$ the fourth suspended in serum-free DMEM containing 2 μg/mL rREG4 after being pre-incubated with 1 μg/mL REG4 mAb, and the fifth was used as control. All of these PANC-1 cells were suspended at a density of 2.0×10^6 cells/mL. The same procedure was applied for ASPC-1 cells.

Transwell migration assays were carried out using 24-well Millicell Hanging Cell Culture inserts with 8 μm PET (Millipore, Boston, MA, USA). The invasion assay was carried out using QCM 24-well cell invasion assay kit (ECM554; Millipore) pre-coated with ECMatrix, a reconstituted basement membrane matrix of proteins derived from the Engelbreth–Holm–Swarm mouse tumor. After the RT-PCR qualification, which suggested a possible correlation between *REG4* and MMP-7, MMP-9, a Transwell invasion assay was carried out to test the effect of MMP inhibitor on REG4-induced enhanced invasion. After pre-incubation with rREG4, PANC-1 cells were treated with MMP antibody or siRNA, then collected for subsequent Transwell invasion assay.

Cells (2.0×10^5) were seeded in the upper chamber. NIH 3T3-fibroblast conditioned medium was added to the lower chamber. After 48 h of incubation at 37°C in 5% CO_2, cells on the upper surface of the inner chamber were removed. Migrating or invading cells that adhered to the lower surface of the membrane were fixed and stained with H&E. The migrating or invading cells were counted at ×400 magnification in 10 different fields for each insert.

Real-time RT-PCR quantification. Five samples of PANC-1 cells were taken. One of them was treated with rREG4, another treated with REG4 antibody, the third treated with CM, the fourth treated with CM_{Ab}, and the last was used as control. The same procedure was applied to ASPC-1 cells. Five samples of BXPC-3 cells were taken. One of them was treated with REG4 antibody, another transfected with *REG4* siRNA (3 μL), the third transfected with *REG4* siRNA (6 μL), and the last two samples were used as blank control and siRNA control. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed using PrimeScript 1st Strand cDNA Synthesis kit (DRR047A; Takara, Osaka, Japan) according to the manufacturer's instructions. The RT-PCR was carried out using the MX3000P system (Stratagene, Santa Clara, CA, USA), using gene-specific primers with SYBR Premix ExTaq Kit (Takara). *GAPDH* was used as an internal control. The primers are shown in Table 1. After 5 min initial denaturation at 95°C, 40 cycles of amplification were carried out at 95°C for 10 s, annealing time (shown in Table 1) for 20 s, and 72°C for 20 s. At the end of the PCR cycles, melting curve analysis was carried out. The relative expression levels were calculated using the $2^{-\Delta Ct}$ method.

Western blot verification. REG4 protein in the pancreatic cancer cells conditioned medium was concentrated using Amicon Ultra Centrifugal Filter NMWL 3000 (Millipore) according to the manufacturer's instructions. Total protein of cells was extracted using RIPA lysis buffer and fractionated by SDS-PAGE. The proteins were electrotransferred onto PVDF membranes and incubated with primary antibodies at 4°C overnight. The primary antibodies included anti-REG4 (1:500; R&D Systems), anti-MMP-7 (1:2000; Epitomics, Burlingame, CA, USA), anti-MMP-9 (1:2000; Epitomics), and anti-β-tubulin (1:10 000; Epitomics). After incubating with secondary antibody (1:5000; Huaan Biotech, Hangzhou, China) for 2 h, the membranes were treated with electrochemiluminescence reagent (Generay, Shanghai, China) and exposed to autoradiographic films.

Gelatin and casein substrate zymography. Conditioned media from PANC-1 and ASPC-1 cells was concentrated using an Amicon Ultra Centrifugal Filter NMWL 3000 (Millipore). All specimens (cellular protein quantity, 20 μg) were treated with sample loading buffer (1.5% SDS, 15% glycerol, and 0.005% bromphenol blue) and loaded onto the gels. Gelatin and casein zymography were carried out to detect MMP-9 and MMP-7, respectively, using an MMP Gelatin Zymography assay kit (GMS30071.1; GenMed, Shanghai, China) and an MMP Casein Zymography assay kit (GMS30071.2; GenMed).

Immunohistochemical staining. The sections were deparaffinized and rehydrated using graded ethanol. Antigen retrieval was carried out by autoclaving for 3 min in 0.01 M citrate buffer (pH 6.0). The sections was blocked with 3% (v/v) H_2O_2 for 10 min followed by 10% (v/v) normal goat serum for 15 min at room temperature. Then the sections were incubated

Table 1. Primers used for PCR analysis of candidate genes examined for their involvement in *REG4*-induced cell invasion

Invasion candidate genes	Sequence	Fragment size	Annealing time
MMP1 (NM_002421.3)	F: GTCAAGTTTGTGGCTTATGGATT R: GAAGAGTTATCCCTTGCCTATCC	220 bp	55°C
MMP9 (NM_004994.2)	F: TAGGGCTCCCGTCCTGCTT R: CCACCTCCACTCCTCCCTTTC	125 bp	58°C
MMP2 (NM_004530.4)	F: TTGACGGTAAGGACGGACTC R: CATACTTCACACGGACCACTTG	126 bp	55°C
MMP3 (NM_002422.3)	F: ACAAGGAGGCAGGCAAGAC R: CCACGCACAGCAACAGTAG	100 bp	58°C
MMP7 (NM_002423.3)	F: GAAACTTCAGGCAGAACATCC R: GAGTGGAGGAACAGTGCTTATC	90 bp	55°C
TIMP2 (NM_003255.4)	F: TGTTCGCTTCCTGTATGGTGAT R: TTCCACTCTGGGTCAAATGC	200 bp	55°C
PLAU (NM_002658.3)	F: CACACACTGCTTCATTGATTACC R: CAAGGCAATGTCGTTGTGGT	169 bp	55°C
Cyclin E1 (NM_001238.1)	F: CAGGGTATCAGTGGTGCGACAT R: TTGCTCGGGCTTTGTCCAG	177 bp	55°C
Cyclin D1 (NM_053056.2)	F: TGAAGGGAGGTGGCAAGAGT R: ATAGCAGCAAACAATGTGAAAGA	215 bp	55°C
MMP10 (NM_002425.2)	F: CCCACTGGAACCCTGAACC R: TATGGATGCCTCTTGGATAACCT	180 bp	55°C
MMP11(NM_005940.3)	F: CCTAAAGGTATGGAGCGATGTG R:CCGATAGTCCAGGTCTCATCATAG	207 bp	55°C
MMP13 (NM_002427.3)	F: GTCTTTCTTCGGCTTAGAGGTG R: TGTCAGCAATGCCATCGTG	272 bp	60°C
TIMP3 (NM_000362.4)	F: CTCTGCTCTGTCCAGGGTAGG R: CTTAGGTAGCCAGAAGCCAAAC	262 bp	55°C
TIMP4 (NM_003256.2)	F: GACTATTCCCTTTCCTCCCCA R: TGTGTATGACATTCGCCATTTCT	85 bp	55°C
GAPDH (NM_002046.3)	F: TGAAGGTCGGAGTCAACGG R: CTGGAAGATGGTGATGGGATT	223 bp	56°C

F, forward; PLAU, plasminogen activator urokinase; R, reverse; TIMP, tissue inhibitor of metalloproteinase.

at 4°C overnight with primary antibody including anti-REG4 (1:100; R&D Systems), anti-MMP-7 (1:250; Epitomics), and anti-MMP-9 (1:200; Epitomics). The sections were then incubated with HRP-conjugated secondary antibody (Zhongshan Biotech, Beijing, China) for 30 min at room temperature. We used DABto visualize the signal development. Finally, the sections were counterstained with hematoxylin.

Immunohistochemical evaluation. The immunohistochemical (IHC) evaluation was independently done by two pathologists without knowledge of the clinical data. The immunoreactivity levels of each case were estimated under light microscope by assessing the average signal intensity (on a scale of 0 to 3) and the proportion of cells showing a positive cytoplasm stain (0, <5%; 1, 5–25%; 2, 26–50%; 1, 51–75%; 4, 76–100%). The intensity and proportion scores were then multiplied to obtain a composite score. A score of 0–3 was defined as negative and a score of 4–12 was categorized as positive.

Statistical analysis. Statistical analyses of the data were carried out using SPSS version 13.0 software. Normally distributed continuous variables were expressed as the mean ± SD; means were compared by either a paired sample *t*-test or one-way ANOVA, as appropriate. Categorical variables were presented as percentages and were analyzed by Fisher's exact test. The relationship between REG4 expression and MMP-7 and MMP-9 was assessed by Spearman's correlation coefficients. All *P*-values were obtained from two-tailed statistical tests. Differences were considered significant when $P < 0.05$.

Results

***REG4* expression in pancreatic cancer cell lines.** The *REG4* mRNA expression levels in three pancreatic cancer cell lines are shown in Figure 1(A). To confirm these results, cell lysates and cell culture medium were analyzed by Western blotting with anti-REG4 antibody (Fig. 1B). Consistent with the RT-PCR results, high levels of REG4 expression were found in BXPC-3 cells and its culture media. In contrast, PANC-1 and ASPC-1 cells had very low level of REG4 expression, and no protein signals were detected in the culture medium.

Effect of REG4 on cell proliferation. The overall results suggest that *REG4* can act as a cell proliferation promoter in pancreatic cancer. After 24 h of treatment, rREG4 and CM were found to significantly promote the proliferation of both PANC-1 and ASPC-1 cells (Fig. 2). After 48 and 72 h of treatment, no further increase in cell number was observed across all groups (Fig. 2, 48 h *vs* 24 h, 72 h *vs* 48 h). Therefore, we propose that the effect of REG4 on cell proliferation is limited to 72 h or even less. Furthermore, no significant dose-dependent effect of rREG4 on cell growth was observed. The effect of REG4 on cell proliferation was significantly lower in CM_{siREG4} and CM_{Ab} treated groups compared with CM treated groups, but was significantly higher compared with the control groups. This result shows that the proliferation effect induced by *REG4* can be reduced by suppressing the expression of *REG4* with REG4 antibody or *REG4* siRNA, but cannot be blocked completely, and the detailed mechanism should be further investigated.

Involvement of *REG4* in pancreatic cancer cell invasion and migration. We further examined the effect of *REG4* on invasion of pancreatic cancer cells using a Transwell invasion assay model. We found that rREG4 and CM significantly promoted the invasion of PANC-1 and ASPC-1 cell lines, but REG4 antibody and CM_{Ab} significantly reduced this potential (Fig. 3). In contrast, the invasive potential of BXPC-3 cells

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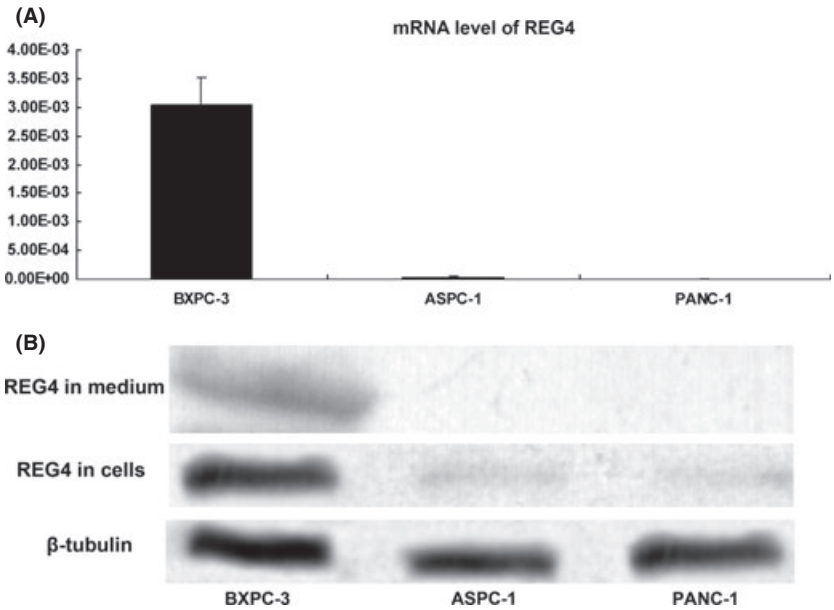

Fig. 1. *REG4* expression in three pancreatic cancer cell lines was analyzed and compared by real-time PCR and Western blotting. (A) Real-time PCR: BXPC-3 cells showed a high *REG4* expression level but ASPC-1 and PANC-1 cells showed a low level of *REG4* expression. (B) Western blotting: BXPC-3 cells and its culture medium showed high levels of *REG4* expression; PANC-1 and ASPC-1 cells had a very low level of *REG4* expression, and no detectable proteins were found in the culture media. β-tubulin was used as an internal control.

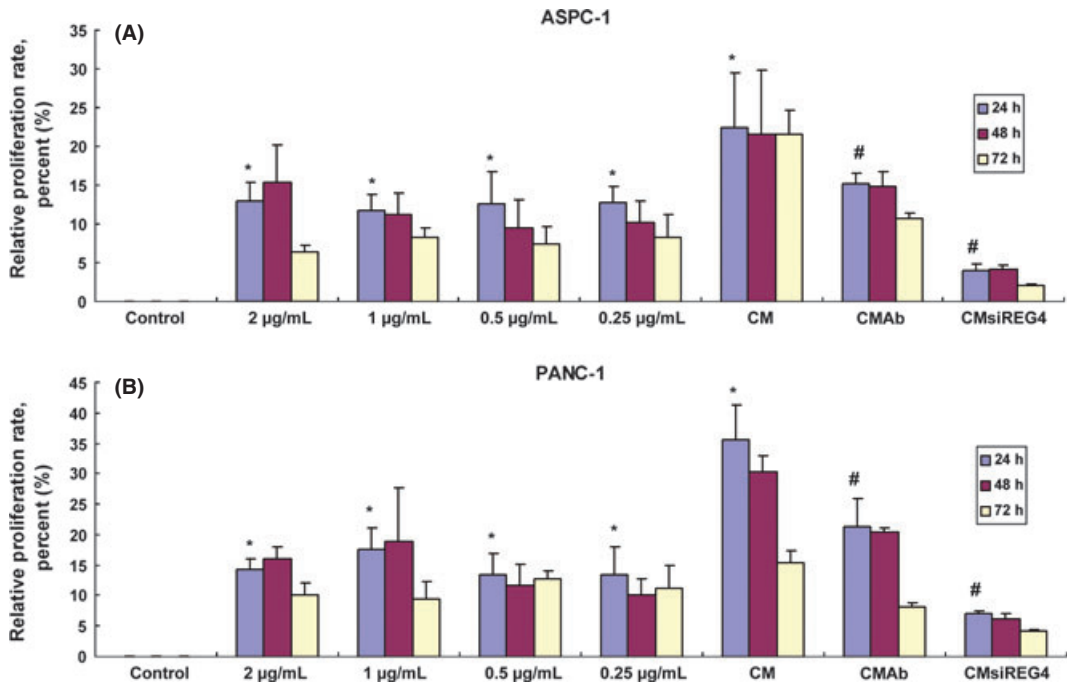

Fig. 2. Effect of *REG4* on cell growth was tested by MTT proliferation assay. *Proliferation rates of (A) ASPC-1 and (B) PANC-1 pancreatic cancer cells treated with conditioned media (CM) and various concentrations of recombinant human REG4 protein were significantly increased compared with the negative control ($P < 0.05$). #Proliferation rates of ASPC-1 and PANC-1 cells treated with CM incubated with REG4 mAb (CM_{Ab}) and CM with *REG4* siRNA (CM_{siREG4}) were significantly decreased compared with the CM treated group ($P < 0.05$).

was significantly reduced by REG4 antibody and *REG4* siRNA compared with negative control (Fig. 4). The above evidence illustrates that REG4 may enhance the invasion ability of pancreatic cancer cells; meanwhile, this invasion potential can be inhibited using *REG4* siRNA or REG4 antibody to downregulate or block *REG4* expressions. A similar effect of REG4 on cancer cell migration was also observed (Fig. 5).

Examination of candidate genes related to *REG4*-induced cell invasion. In order to identify genes related to *REG4*-induced cell invasion in pancreatic cancer, RT-PCR analysis was carried out. Among the candidate genes, expression levels of *MMP-7* and *MMP-9* were remarkably elevated in ASPC-1 and PANC-1 cells treated with rREG4 and CM (Fig. 6). To further verify the relationship between *REG4* expression and invasion-related candidate genes, samples from BXPC-3, a cell line with elevated *REG4* expression, were selected. Some of the samples were incubated with REG4 antibody while the rest were transfected with *REG4* siRNA. The mRNA and protein levels of MMP-7 and MMP-9 were found to be significantly reduced compared with those of the control cells (Fig. 7). However, there were no differences in the expression of *MMP-1, MMP-2, MMP-3, MMP-10, MMP-11, MMP-13, TIMP-2,*

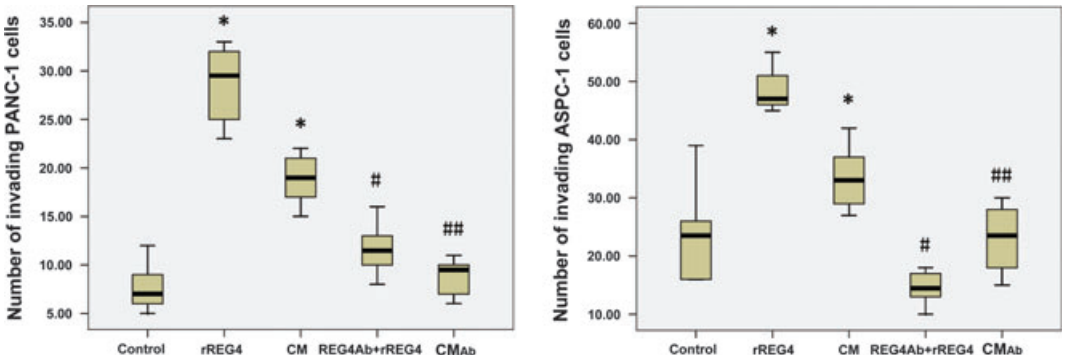

Fig. 3. Invasion ability of ASPC-1 and PANC-1 pancreatic cancer cells was analyzed in a Transwell invasion assay. *Cells treated with recombinant human REG4 protein (rREG4) and conditioned media (CM) showed significantly higher invasive properties compared with control cells ($P < 0.05$). #Cells treated with rREG4 followed by REG4 antibody (REG4Ab) compared with the rREG4 treated group ($P < 0.05$). ##Cells treated with CM incubated with REG4 mAb (CM_{Ab}) compared with the CM treated group ($P < 0.05$).

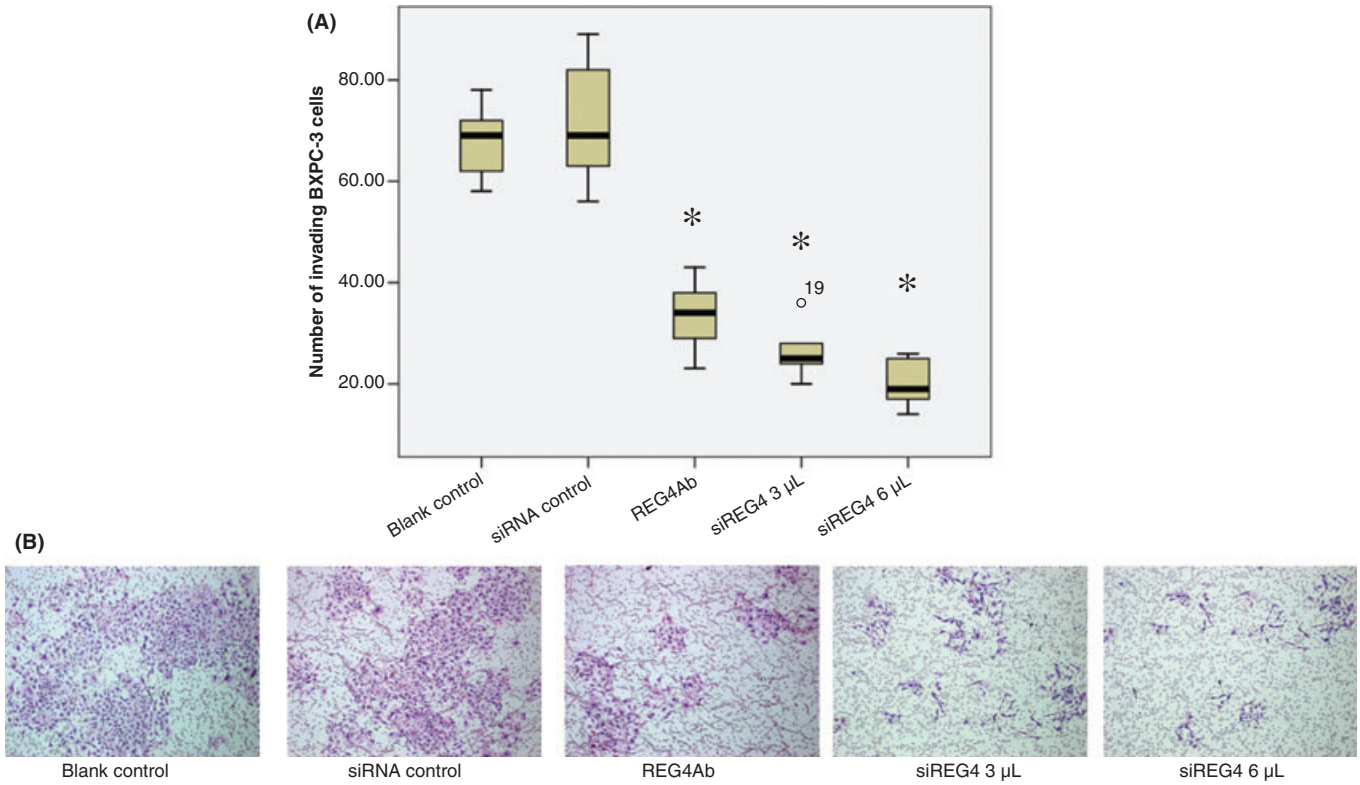

Fig. 4. Transwell invasion assay of BXPC-3 pancreatic cancer cells. REG4 antibody (REG4Ab) and REG4 siRNA (siREG4) treated cells showed lower invasive properties compared with control cells (*$P < 0.05$). (A) Data are expressed as the mean number of invading cells per field (average of 10 fields per filter). (B) Micrographs showing cells invading through 8-μm pores on the lower side of the filters.

TIMP-3, TIMP-4 or *PLAU* (data not shown). We further tested the involvement of MMP-7 and MMP-9 in *REG4*-induced enhanced invasion of human pancreatic cancer cells using MMP antibody or siRNA, and we found that ASPC-1 and PANC-1 cells treated with MMP-7 or MMP-9 antobody or siRNA after pre-incubateion with rREG4 significantly reduced the invasive potential of cancer cells (Fig. 8). Taken together, these results suggest that the promotional effect of *REG4* on pancreatic cancer cell invasion may be associated with the expression level of MMP-7 and MMP-9.

Detection of metalloproteinase activity. Using gelatin zymography, PANC-1 and ASPC-1 cells treated with rREG4 showed far higher levels of the active form of MMP-9 compared to the control groups. Similarly, casein zymography revealed a far higher level of the active form of MMP-7 in PANC-1 and ASPC-1 cells treated with rREG4, compared to the control groups (Fig. 9).

Correlation between *REG4* and MMP-7 and MMP-9 expression in pancreatic cancer. To confirm the correlation between *REG4* and MMP-7 and MMP-9 expression, IHC was used in 40 pancreatic cancer tissues. Immunohistochemical staining showed the brown signal of *REG4* expression in the tumor cells. Clinicopathological analyses also showed that *REG4* expression is associated with lymph node metastasis and vascular invasion (Table 2).

We also tested MMP-7 and MMP-9 expression in the 40 pancreatic cancer tissues. There was a positive correlation

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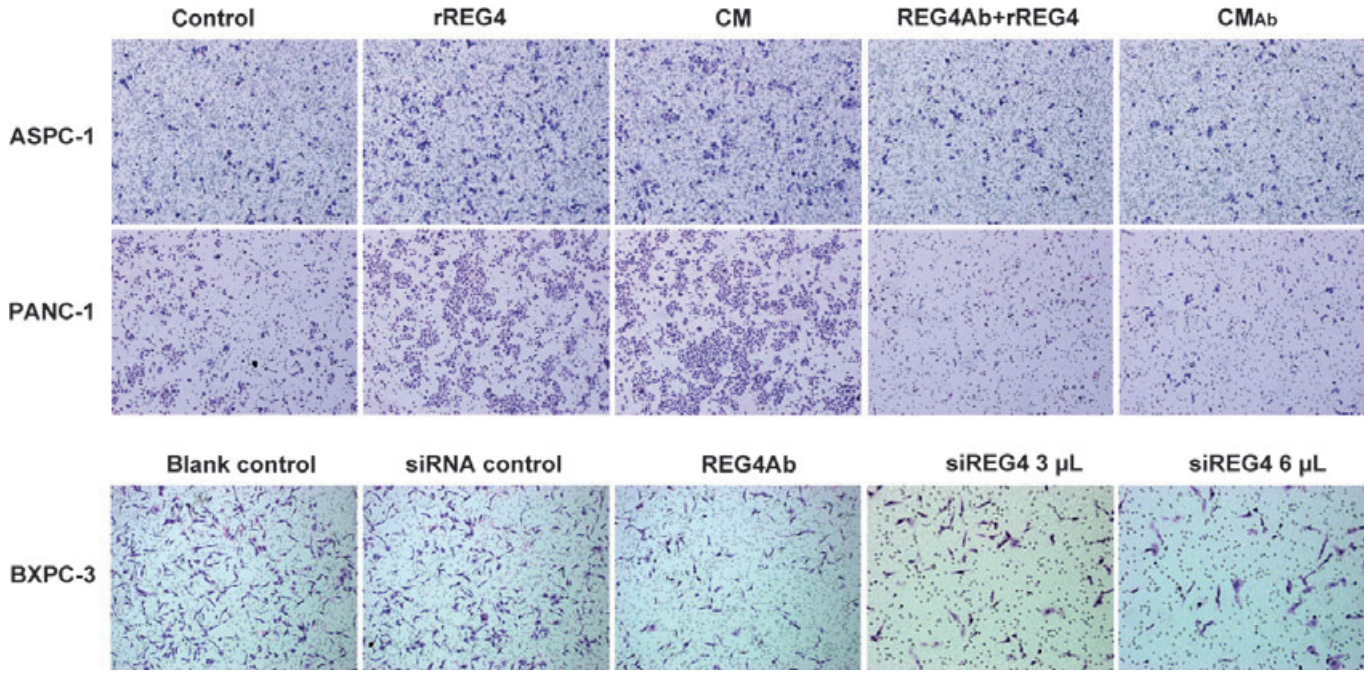

Fig. 5. Transwell migration assay of ASPC-1 and PANC-1 pancreatic cancer cells. Cells treated with recombinant human REG4 protein (rREG4) and conditioned media (CM) showed significantly higher migration properties compared with control cells. Cells treated with rREG4 followed by REG4 antibody (REG4Ab) showed reduced mobility compared with the rREG4 treated group. Cells treated with CM incubated with REG4 mAb (CM_{Ab}) showed reduced mobility compared with the CM treated group. Cells treated with REG4Ab and REG4 siRNA (siREG4) showed lower migration properties compared with control cells in BXPC-3 cells.

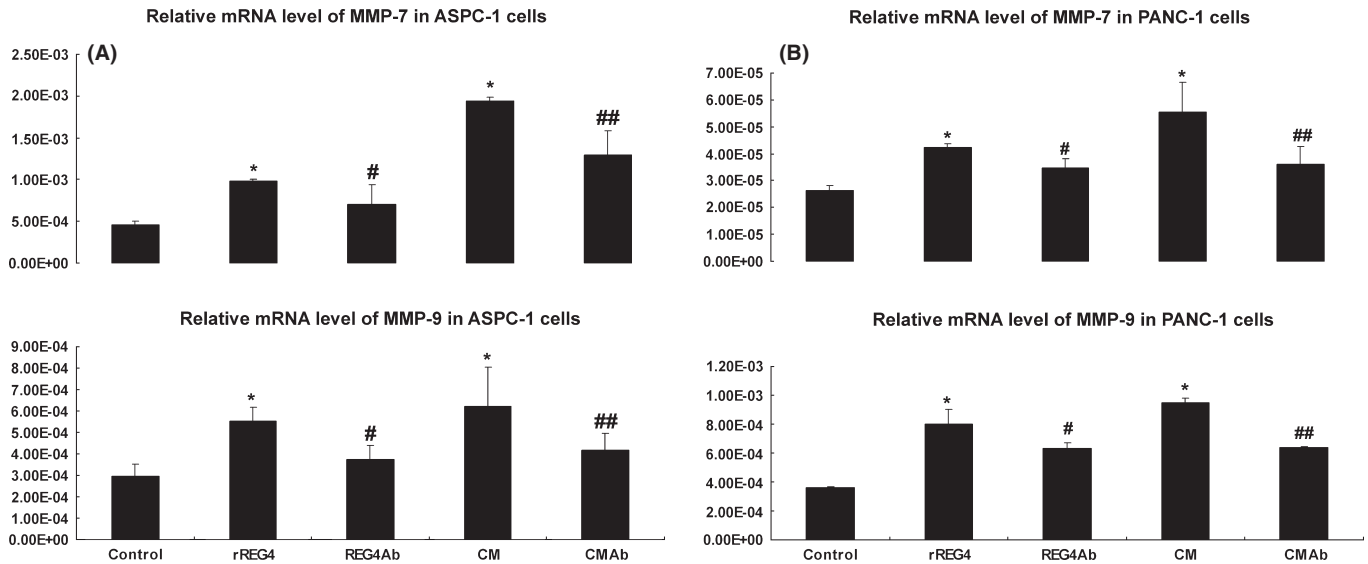

Fig. 6. Expression levels of *MMP-7* and *MMP-9* induced by *REG4* were analyzed in ASPC-1 and PANC-1 pancreatic cancer cells. **MMP-7* and *MMP-9* expression was upregulated when treated with recombinant human REG4 protein (rREG4) and conditioned media (CM) ($P < 0.05$). #REG4 antibody (REG4Ab) treated cells compared with rREG4 treated groups ($P < 0.05$). ##Cells treated with CM incubated with REG4 mAb (CM_{Ab}) compared with CM treated groups ($P < 0.05$). (A) ASPC-1 cells; (B) PANC-1 cells.

between the levels of REG4 and MMP-7 ($R = 0.545$, $P = 0.001$, Spearman's ρ-test). Similarly, a positive correlation was found between the levels of *REG4* and MMP-9 ($R = 0.471$, $P = 0.007$, Spearman's ρ-test) (Table 3, Fig. 10). In order to further clarify the role of *REG4* in pancreatic cancer, we also examined REG4 levels in the peritumoral pancreatic tissues and found that REG4 showed very weak expression (Fig. 11A). Hematoxylin–eosin staining was used for pathological diagnosis of tumor (Fig. 11B); negative control was also used to test for the specificity of the antibody involved (Fig. 11C). The production of REG4, MMP-9, and MMP-7 were also detected in the liver metastatic foci of pancreatic cancer, where they showed strong positive expression (Fig. 11D–F).

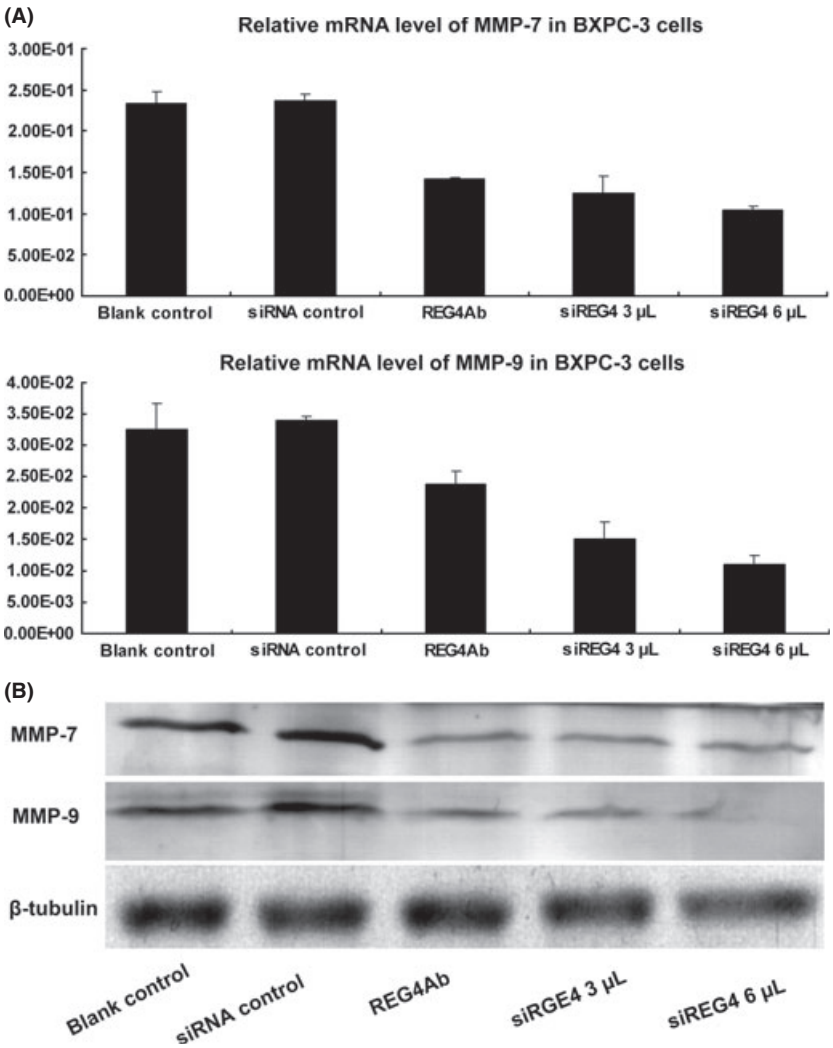

Fig. 7. *REG4* induced *MMP-7* and *MMP-9* expression in BXPC-3 pancreatic cancer cells. *MMP-7* and *MMP-9* were found to be significantly reduced at both the mRNA and protein level when incubated with REG4 antibody (REG4Ab) or transfected with *REG4* siRNA (siRGE4) in BXPC-3 cells. (A) Real-time PCR; (B) Western blotting.

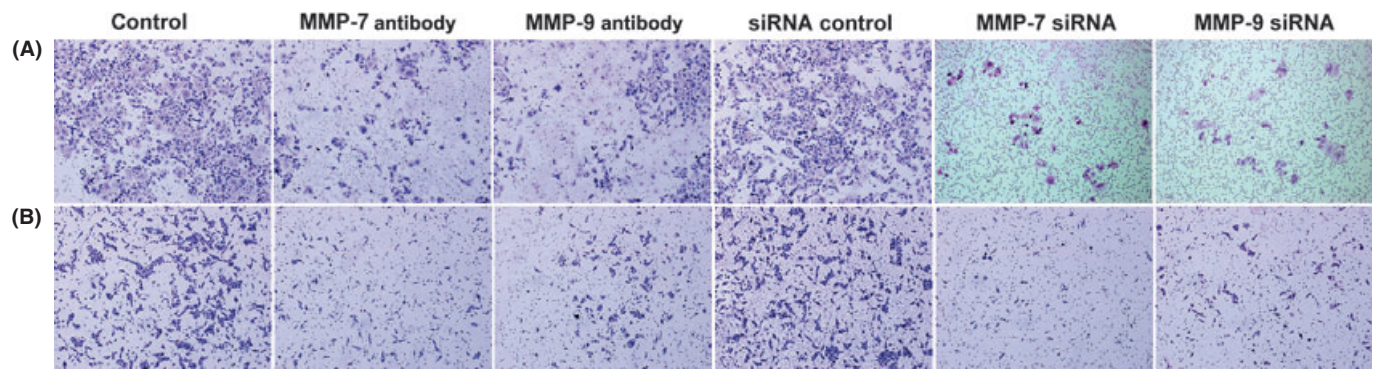

Fig. 8. Effect of MMP-7 and MMP-9 inhibitor or siRNA on *REG4*-induced enhanced invasion. The MMP-7 or MMP-9 inhibitor or siRNA significantly reduced *REG4*-induced invasive potential. (A) ASPC-1; (B) PANC-1.

Discussion

Recent studies have shown that *REG4* is involved in digestive tract malignancies including gastric,[5] colorectal,[9,14] and pancreatic cancer,[7,22] and its overexpression is associated with the initiation and progression of pancreatic cancer.[7,23] Our previous research on pancreatic cancer also suggested that the serum level of REG4 is higher in pancreatic cancer patients than in patients with benign pancreatic disease and healthy controls, and concurrent testing of REG4 and CA199 can increase the diagnostic sensitivity to 90.5%.[12] In this study, we confirmed that both pancreatic cancer cell BXPC-3 and its culture media had high levels of *REG4* expression, indicating that pancreatic cancer cell may produce and secrete REG4 to influence tumor initiation and development. We also examined REG4 protein levels in the peritumoral pancreatic

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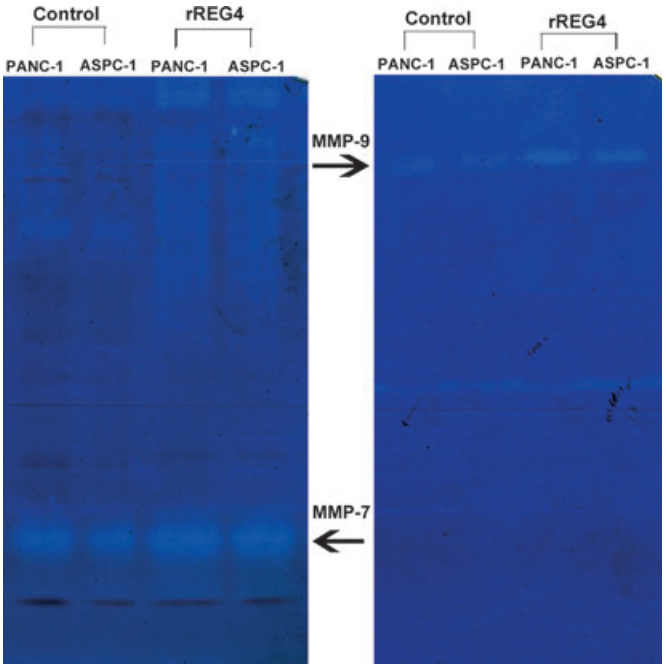

Fig. 9. Zymography analysis of MMP activity. The group treated with recombinant human REG4 protein (rREG4) showed higher levels of active *MMP-7* and *MMP-9* compared to the control groups.

Table 2. *REG4* expression in 40 pancreatic cancers and its clinicopathological significance

Parameters	Total	*REG4* expression			*P*-value
		Negative	Positive	Expression ratio (%)	
Gender					
Female	18	4	14	77.78	1.0000
Male	22	6	16	72.73	
Differentiation					
Well	24	6	18	75.00	1.0000
Poorly	16	4	12	75.00	
Lymph node metastasis					
Yes	18	0	18	100.00	0.0010
No	22	10	12	54.55	
T factor					
T1	8	3	5	62.50	0.7000
T2	22	5	17	77.30	
T3	10	2	8	80.00	
TNM stage					
I + II	23	7	16	69.60	0.4710
III + IV	17	3	14	82.40	
Distant metastasis					
Yes	16	1	15	93.80	0.0320
No	24	9	15	62.50	
Vascular invasion					
Yes	20	0	20	100.00	0.0004
No	20	10	10	50.00	

tissues and found that the tissues showed very weak expression of REG4. So we proposed that REG4 might act in a paracrine manner to influence inflammation and tumor initiation. Our results support the opinion that REG4 can be used as a tumor-specific serum marker. Takayama *et al.*[7] also revealed that serum REG4 has potential as a screening serum marker for

Table 3. Correlation between *REG4* and *MMP-7* and *MMP-9* in pancreatic cancer

	REG4		*R*	*P*-value
	Positive	Negative		
MMP-7				
Positive	24	2	0.545	0.001
Negative	6	8		
MMP-9				
Positive	16	0	0.471	0.007
Negative	14	10		

pancreatic cancer diagnosis, including early stage disease. However, in pancreatic cancer cell lines PANC-1 and ASPC-1, *REG4* expression was very low, and no detectable protein was found in the culture medium. Hence, we propose that *REG4* expression may be related to cell properties. Tamura *et al.*[8] also found that *REG4* expression is more frequently observed in well to moderately differentiated gall bladder carcinomas than in poorly differentiated ones.

In order to clarify the role of REG4 on the growth of pancreatic cancer cells, we chose PANC-1 and ASPC-1 cells, which had very low REG4 expression levels, to test the effect of REG4 on cell proliferation. We found that rREG4 and BXPC-3 CM significantly stimulated the proliferation of both PANC-1 and ASPC-1 cells. Compared with the CM treatment group, the proliferation effect was significantly lower in CM_{siREG4} and CM_{Ab} treatment groups. These results suggest that *REG4* may promote cell proliferation in pancreatic cancer. A study on adenoid cystic carcinoma also suggested that REG4 might accelerate cell growth.[24] In our present study, no significant dose-dependent effect of REG4 on pancreatic cancer cell growth was observed. Rafa *et al.*[13] also found that REG4 had a similar effect on colon cancer cells. As REG4 might act in an autocrine or paracrine manner to stimulate the growth of cells, depending on the target cell, we propose that the regulatory mechanisms that interact with REG4 intracellularly or extracellularly might restrict the stimulation of growth induced by REG4.

Cell migration and invasion abilities are important indicators for malignancy and metastasis of tumor cells. *REG4*, an oncogene, may be potentially involved in invasion, metastasis, and carcinogenesis.[5] In this study, we examined the effect of REG4 on the invasion and migration of pancreatic cancer cells using a Transwell assay. We found that rREG4 and BXPC-3 CM significantly promoted migration and invasion of pancreatic cancer cells, whereas CM_{siREG4} and CM_{Ab} significantly reduced this potential. The result indicates that REG4 may promote migration and invasion of pancreatic cancer cells.

The MMP family, which causes tumor invasion and formation of distant metastases, is known to play a key role in various human cancers including pancreatic cancer.[25] Crawford *et al.*[26] have shown that MMP-7 is expressed in the majority of human pancreatic adenocarcinomas, but is never detected in the normal pancreas. Other studies have indicated that expression of MMP-7 correlates significantly with infiltrative growth pattern, and lymph node and liver metastasis of pancreatic carcinoma,[27,28] and also markedly increases invasion of pancreatic cancer cells *in vitro*.[29] Matsuyama *et al.*[30] reported that pancreatic ductal carcinomas with metastases show much higher MMP-9 expression than metastasis-free carcinomas. Similarly, Nagakawa *et al.*[31] found a major role of MMP-9 in cancer cell infiltration of blood vessels. In the present study, we have attempted to identify genes that are induced by the expression of *REG4* and are thus related to

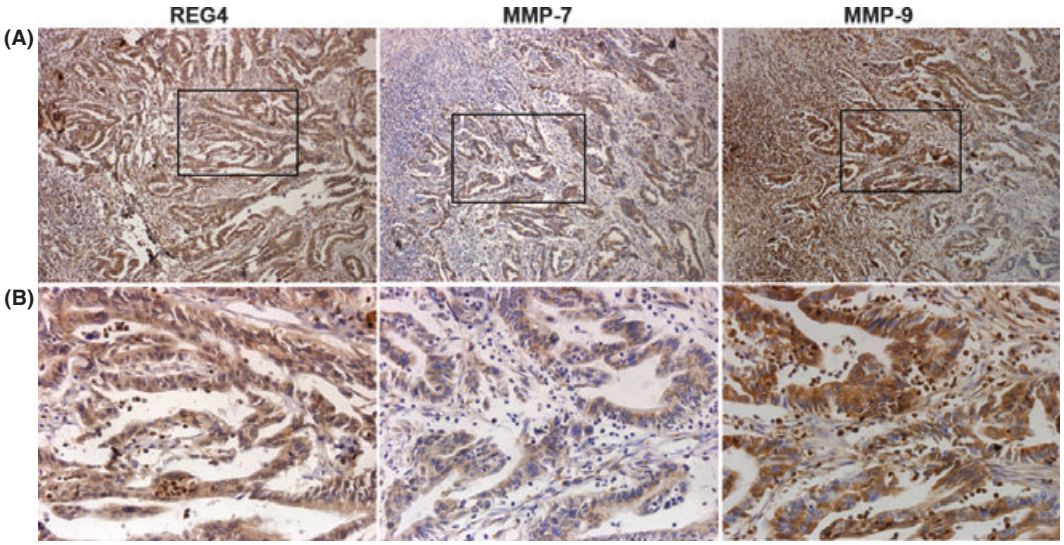

Fig. 10. Immunohistochemical analysis of REG4, MMP-7, and MMP-9 in pancreatic cancer. Positive immunohistochemical staining of REG4, MMP-7, and MMP-9 was mainly in the cytoplasm. (A) Original magnification, ×100. (B) Original magnification, ×400.

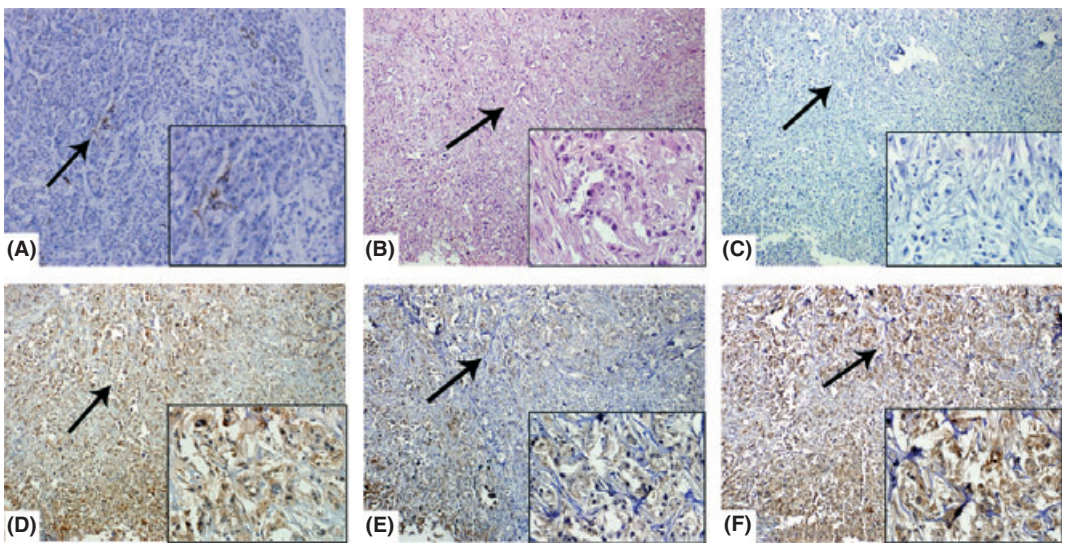

Fig. 11. Immunohistochemical analysis of *REG4* in peritumoral pancreatic tissues. The insert panes show the section indicated by the arrows at a magnification of × 400. (A) *REG4* showed weak expression (↑) in the peritumoral pancreatic tissues; original magnification, ×200. (B) H&E staining of liver metastatic foci of pancreatic cancer; original magnification, ×100. (C) Negative control of immunohistochemistry of liver metastatic foci of pancreatic cancer; original magnification, ×100. (D) *REG4* showed strong positive expression (↑) in liver metastatic foci of pancreatic cancer. (E) MMP-7 showed strong positive expression (↑) in liver metastatic foci of pancreatic cancer. (F) MMP-9 showed strong positive expression (↑) in liver metastatic foci of pancreatic cancer.

invasion. Real-time RT-PCR and Western blot analysis of several candidate genes was carried out using rREG4, as well as CM-treated ASPC-1 and PANC-1 cells. In candidate genes related to cancer invasion, we found that the invasion-related candidate genes *MMP-7* and *MMP-9* were remarkably elevated in ASPC-1 and PANC-1 cells treated with rREG4 and BXPC-3 CM, respectively; in contrast, *MMP-7* and *MMP-9* were significantly reduced at both the mRNA and protein level when treated with REG4 antibody or transfected with *REG4* siRNA. Furthermore, MMP-7 and MMP-9 antibody and siRNA reduced *REG4*-induced enhanced invasive potential. Analysis using IHC in pancreatic cancer tissues further confirmed that *REG4* expression correlates with MMP-7 and MMP-9. Strong positive expression of REG4, MMP-9, and MMP-7 in liver metastatic foci of pancreatic cancer also confirmed the above results.

These results suggest that *REG4* may promote pancreatic cancer cell invasion through MMP-7 and MMP-9. *REG4* over-expression in tumor cells has been associated with cell growth and adhesion.[4,19,24] Bishnupuri *et al.* also found that the expression of *REG4* may be a potent activator of the EGFR/Akt/AP-1 signaling pathway in colon cancer cells.[32] Based on the above results, we propose that MMP-7 and MMP-9 upregulation may be induced by REG4 through a growth factor such as vascular endothelial growth factor and cell adhesion molecules, and additional studies are required to determine the molecular mechanisms underlying *REG4*-induced MMP-7 and MMP-9 upregulation.

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In conclusion, our results show that overexpression of *REG4* not only stimulates cell growth, but also promotes *in vitro* invasiveness of pancreatic cancer cells. Both MMP-7 and MMP-9 are closely involved in the invasion induced by *REG4* in pancreatic cancer. Our results also support the opinion that REG4 can be used as a tumor-specific serum marker for invasion and metastasis. Identification of these signaling pathways will further improve our understanding of the molecular mechanism of REG4-induced invasion in pancreatic cancer, and help devise novel techniques to diagnose and treat pancreatic cancer.

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Disclosure Statement

The authors have no conflict of interest.

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