Novel tumor marker REG4 detected in serum of patients with resectable pancreatic cancer and feasibility for antibody therapy targeting REG4

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Pancreatic ductal adenocarcinoma (PDAC) shows the worst mortality rate among common malignancies, with a 5-year survival rate of only 4%, and the majority of PDAC patients are diagnosed at an advanced stage in which no effective therapy is available at present. Although the proportion of curable cases is still not so high, surgical resection of early stage PDAC is the only way to cure the disease. Hence, establishment of a screening strategy to detect early stage PDAC by novel serological markers is required urgently, and development of novel molecular therapies for PDAC treatment is also eagerly expected. We here report overexpression of REG4, a new member of the regenerating islet-derived (REG) family, in PDAC cells on the basis of genome-wide cDNA microarray analysis as well as reverse transcription–polymerase chain reaction and immunohistochemical analysis. We also detected significant elevation of REG4 in the serum of some patients with early-stage PDAC using our enzyme-linked immunosorbent assay system, indicating the possibility of REG4 as a new serological marker of PDAC. Furthermore, we found that knockdown of endogenous REG4 expression in PDAC cell lines with small interfering RNA caused a decrease in cell viability. Concordantly, addition of recombinant REG4 to the culture medium enhanced growth of a PDAC cell line in a dose-dependent manner. A monoclonal antibody against REG4 neutralized its growth-promoting effects and attenuated significantly the growth of PDAC cells. These findings indicate that REG4 is a promising tumor marker to screen early-stage PDAC, and also that neutralization of REG4 by the antibody may offer novel potential tools for the treatment of PDAC. (*Cancer Sci* 2006; 97: 1191–1197)

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the western world and shows the worst mortality among common malignancies, with a 5-year survival rate of only 4%.[1,2] In 2006, it is estimated that approximately 33 730 new cases of pancreatic cancer will be diagnosed in the USA, with 32 300 of these people expected to die of the disease.[3] In Japan nearly 20 000 PDAC patients die each year. Because the majority of PDAC patients are diagnosed at an advanced stage, no effective therapy to cure the disease is available at present. Several approaches that combine surgery with chemotherapy, including 5-fluorouracil (5-FU) or gemcitabine, with or without radiation, may improve patients' quality of life,[1,2] but those treatments have a very limited effect on long-term survival of PDAC patients due to its extremely aggressive and chemoresistant nature.

The very poor prognosis of PDAC arises from several reasons, including the difficulty of detecting PDAC at an early stage.[1,2] Despite improvements in diagnostic imaging techniques, such as endoscopic ultrasound and magnetic resonance cholangiopancreatography,[1,2] most patients do not undergo imaging procedures because they do not have any symptoms until late in the course of the disease. An accurate and easy serological test, such as for prostate-specific antigen (PSA) for prostate cancer, could facilitate detection of PDAC at an early stage and could be applied for mass-screening of PDAC. Surgical resection of early-stage PDAC can offer the relatively favorable prognosis of 50–60% 5-year survival.[2] Hence, considering the biological aggressiveness and resistance to chemotherapy of PDAC, one of the most realistic strategies to improve the prognosis of this fatal disease is to screen PDAC at an early stage or to screen high-risk individuals using a non-invasive serological test. Currently CA19-9 is the only reliable serological marker for PDAC, but it has poor detection and discrimination values because: (i) approximately 10–15% of individuals do not secrete CA19-9 due to their Lewis antigen status; (ii) it is not specific to pancreatic cancer and is also elevated in benign conditions (e.g. pancreatitis, cholangitis, cirrhosis); and (iii) it is usually within a normal range in PDAC patients at an early stage.[4,5] Hence, establishment of a screening strategy through the development of a novel tumor maker that is more specific and more sensitive to PDAC is required urgently.

Previously, we generated precise genome-wide gene expression profiles of PDAC cells by combining our genome-wide cDNA microarrays with laser beam microdissection.[6] Among the numerous *trans*-activated genes in PDAC cells, in this report we focus on REG4, a new member of regenerating islet-derived (REG) family,[7] as a tumor marker of PDAC. The molecules belonging to the REG family are secreted proteins that play a role in tissue regeneration and inflammation in digestive organs.[7–9] The expression levels of the members were reported to be up-regulated in several gastrointestinal cancers and to function as trophic or anti-apoptotic factors in cancers.[9,10] In the present study, we validated the overexpression of REG4 in PDAC cells by immunohistochemical analysis using anti-REG4 antibody, and established an enzyme-linked immunosorbent assay (ELISA) system to measure REG4 in the serum of patients with PDAC. In addition, we also evaluated the feasibility of antibody therapy neutralizing secreted REG4, which was proven to promote cancer cell growth in an autocrine/paracrine manner. Our results indicate the possibility of using REG4 as a new tumor marker of PDAC as well as the feasibility of using anti-REG4 antibody for molecular-targeting therapy.

Materials and Methods

Clinical samples. Pre-operative and post-operative (3 or 4 weeks after curative resection) serum samples were obtained with informed consent from 11 patients who underwent curative resection for

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pancreatic ductal adenocarcinoma at the Osaka Medical Center for Cancer and Cardiovascular Diseases. Conventional paraffin-embedded tissue sections of PDAC were also obtained from surgical specimens that had been resected at the same center. Tissue microarray samples, where 31 PDAC tissues and two endocrine-tumor tissues were spotted in duplicate, were obtained from ISU ABXIS (Seoul, Korea).

Cell lines. The PDAC cell lines PK-45P and SUIT-2 were provided by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan) and Kyushu Medical Center (Fukuoka, Japan), respectively. MIAPaCa-2 was purchased from American Type Culture Collection (Rockville, MD, USA). PK-45P and SUIT-2 were grown in RPMI-1640 (Sigma-Aldrich, St Louis, MO, USA), and MIAPaCa-2 was grown in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Cansera International, Ontario, Canada) and 1% antibiotic/antimycotic solution (Sigma-Aldrich). Cells were maintained at 37°C in humidified air with 5% CO_2. FreeStyle 293 cells (Invitrogen, Carlsbad, CA, USA) were suspended in FreeStyle 293 Expression Medium (Invitrogen) and were grown in a flask rotated on an orbital shaker platform at 125 r.p.m. Cells were maintained at 37°C in an atmosphere of humidified air with 8% CO_2.

Semi-quantitative reverse transcription–polymerase chain reaction for REG4. Purification of PDAC cells and normal pancreatic ductal epithelial cells was described previously.[6] RNA from the purified cell populations and from normal human heart, lung, liver, kidney, brain and pancreas (BD Biosciences, Palo Alto, CA, USA) were subjected to two rounds of amplification by T7-based *in vitro* transcription (Epicentre Technologies, Madison, WI, USA). We prepared appropriate dilutions of each single-stranded cDNA for subsequent polymerase chain reaction (PCR) amplification by monitoring α-tubulin (*TUBA*) as a quantitative control. The primer sequences were as follows: 5′-AAGGATTATGAGGA-GGTTGGTGT-3′ and 5′-CTTGGGTCTGTAACAAAGCATTC-3′ for *TUBA*; 5′-CCAATTGCTATGGTTACTTCAGG-3′ and 5′-GAAAAACAAGCAGGAGTTGAGTG-3′ for *REG4*. All reactions involved initial denaturation at 94°C for 2 min followed by 23 cycles (for *TUBA*) or 28 cycles (for *REG4*) at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, on a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster, CA, USA).

Antibody generation for REG4 and ELISA. The expression vector carrying His-tagged full-length human REG4 was transfected into 293T cells, and recombinant REG4 (REG4-His) was purified from its culture media with the use of a TALON Purification Kit (Clontech, San Diego, CA, USA). The REG4-His protein was prepared for injection by emulsifying the antigen solution with Freund's complete adjuvant (Medical and Biological Laboratories). Anti-REG4 polyclonal antibody (pAb) was raised in rabbits against the REG4-His protein (Medical and Biological Laboratories, Nagoya, Japan), and the immune sera was purified on affinity columns according to standard protocols. Mouse monoclonal antibody (mAb) was also raised by inoculating REG4-His into BALB/c mice with Freund's complete adjuvant. Their lymphocytes were fused with myeloma P3U1 cells and monoclonal hybrid cells were generated and validated using standard techniques. Serum levels of REG4 were measured by standard sandwich ELISA using these antibodies. Briefly, a 96-well immuno-module microplate (Nalgen Nunc International, Rochester, NY, USA) was precoated with mAb to REG4 (clone 21-1) by incubation overnight at 4°C and blocking for 2 h at room temperature. Five-fold diluted sera was reacted with biotinylated anti-REG4 pAb for 15 min and added to the assay plate precoated with anti-REG4 mAb. After incubation for 2 h, they were washed five times to remove any unbound antibody enzyme reagent, and 8000-fold diluted horseradish peroxidase (HRP)-labeled streptavidin (Amersham, Piscataway, NJ, USA) was reacted for 1 h. After washing five times, TMB substrate solution (Moss, Pasadena, ML, USA) was added to the wells and allowed to react for 15 min. The reaction was stopped by adding 100 μL of 0.18 M sulfuric acid into each well. Color intensity was determined by a photometer at a wavelength of 450 nm with a reference wavelength of 620 nm.

Immunohistochemical staining. The sections were deparaffinized and autoclaved for 15 min at 108°C in citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched by incubation for 30 min in 0.33% hydrogen peroxide diluted in methanol. After incubation with fetal bovine serum (FBS) for blocking, the sections were incubated with anti-REG4 pAb for 1 h at room temperature (1:1000). After washing with phosphate-buffered saline, immunodetection was carried out with peroxidase-labeled antimouse immunoglobulin (Envision Kit; Dako Cytomation, Carpinteria, CA, USA). Finally, the reactants were developed with 3,3′-diaminobenzidine (Dako Cytomation) and the cells were counterstained with hematoxylin.

Small interfering RNA-expressing vector and colony formation assay/MTT assay. To knock down endogenous *REG4* expression in PDAC cells, we used the psiU6BX3.0 vector for expression of short hairpin RNA against a target gene, as described previously.[11] The target sequences of the synthetic oligonucleotides for small interfering RNA (siRNA) for *REG4* were as follows: REG4-si1, 5′-CAGGAGTCCTGGGTGATAT-3′ REG4-si2, 5′-GACAGAA-GGAAGAAACTCA-3′ and EGFPsi, 5′-GAAGCAGCACGAC-TTCTTC-3′ (as a negative control). The PDAC cell lines SUIT-2 (REG4-positive) and MIAPaCa-2 (REG4-negative) were plated onto six-well plates, and transfected with plasmid designed to express siRNA (10 μg) using FuGENE6 (Roche, Basel, Switzerland) according to the manufacturer's instructions. Cells were selected using 0.9 mg/mL (for SUIT-2) or 0.8 mg/mL (for MIAPaCa-2) geneticin (Sigma-Aldrich) for 7 days, and then harvested to analyze the knockdown effect on *REG4* expression. For colony formation assay, transfectants expressing siRNA were grown for 7 days in medium containing geneticin. After fixation with methanol, transfected cells were stained with 0.1% of crystal violet solution to assess colony formation. In MTT assay, cell viability was quantified using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). After 7 days of culture in the geneticin-containing medium, the solution was added at a final concentration of 10%. Following incubation at 37°C for 1.5 h, absorbance was measured at 490 nm and at 630 nm as a reference with a Microplate Reader 550 (Bio-Rad, Hercules, CA, USA).

Generating bioactive recombinant human REG4. To create the bioactive form of REG4, the entire coding sequence of *REG4* cDNA was amplified by PCR using the primer pair 5′-CGGAATTCATGGCTTCCAGAAGCATGC-3′ (forward) and 5′-ATAAGAATGCGGCCGCTGGTCGGTACTTGCACAGG-3′ (reverse), which contained *Eco*RI and *Not*I restriction sites indicated by the first and second underlines, respectively. The product was inserted into the *Eco*RI and *Not*I sites of pCAGGS for expressing a hemagglutinin (HA)-tagged protein. FreeStyle 293 cells were seeded at 1.5×10^5 cells/mL in 30 mL medium. REG4-HA/pCAGGS vectors were transfected with cells using FuGene 6, according to the instruction manual. Culture medium was harvested after 48 h and recombinant human REG4 (rhREG4) was purified with HA agarose (Sigma-Aldrich).

Autocrine assay and antibody neutralizing assay. PK-45P cells were seeded onto 24-well microtiter plates (3×10^3 cells/well). After 2 days, the medium was changed to serum-free medium. The next day, the culture medium was changed to medium containing 0, 0.1, 1 or 10 nM rhREG4 containing 1% FBS, and cells were incubated for 0, 1, 2, 3, 4 or 5 days. At each time point, cell viability was determined by MTT assay as described above. For the antibody neutralizing assay, PK-45P and SUIT-2 cells were seeded onto 24-well microtiter plates (3×10^3 cells/well and 2×10^3 cells/well, respectively). After 2 days, the medium was changed to serum-free medium. The cells were cultured for a further 6 days in medium containing 1% FBS. In the PK-45P

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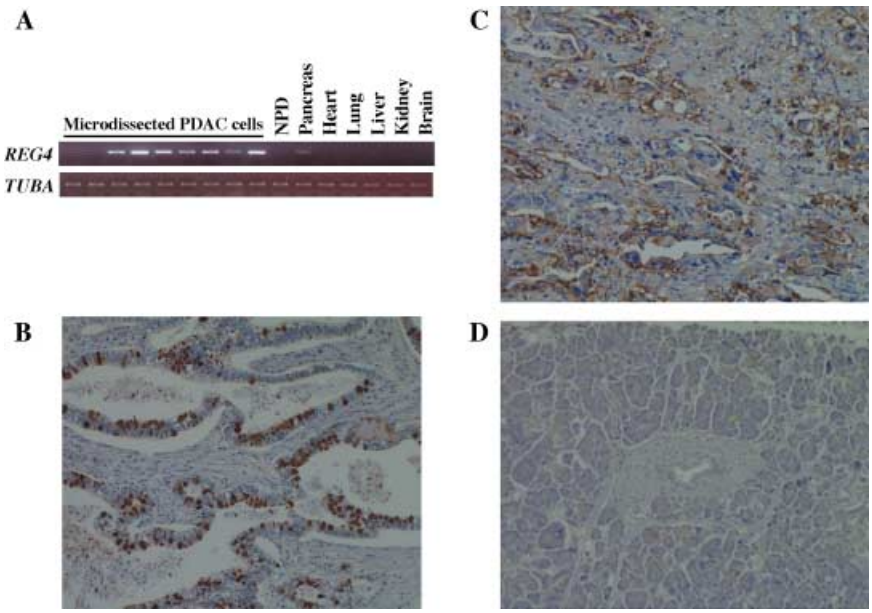

Fig. 1. REG4 mRNA and protein expression levels in pancreatic ductal adenocarcinoma (PDAC) cells. (A) Reverse transcription–polymerase chain reaction analysis of *REG4* and *TUBA* (as a quantitative control) in the microdissected PDAC cells (lanes 1–9) compared with normal pancreatic ductal epithelial cells (NPD), which were also microdissected, normal pancreas and normal vital organs including heart, lung, liver, kidney and brain. (B–D) In the immunohistochemical study using anti-REG4 antibody, intense staining was observed in PDAC cells. Positive staining of REG4 was observed as goblet-like cytoplasmic granules, suggesting secretion of REG4 (B), and at the cytoplasmic membrane (C). In normal pancreatic tissue, acinar cells showed very faint staining, but not in normal ductal epithelium cells and islet cells (D).

medium, 10 nM rhREG4 with or without 100 μg/mL anti-REG4 mAb was added to analyze its neutralizing activity against rhREG4. In the SUIT-2 medium, various concentrations of anti-REG4 mAb were added to analyze its neutralizing activity against endogenous REG4. Mouse anti-WFDC2 monoclonal antibody, which was generated and purified by the same method, was used as a negative control. Cell viability was determined by MTT assay as described above.

Immunoprecipitation. SUIT-2 cells cultured in a 10-cm dish were washed and further cultured for 2 days in serum-free medium. After centrifugation at 10 000*g* and 4°C for 15 min, the supernatant was treated with protein G sepharose (Zymed Laboratories, San Francisco, CA, USA) for 1 h at 4°C. The pretreated supernatant was then added to a mixture of protein G sepharose that was preincubated with anti-REG4 mAb. Incubation was carried out with gentle rotation at 4°C for 4 h followed by two washing steps. Bound proteins were eluted and separated by 15% sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoretic separation, proteins were transferred to nitrocellulose membranes (Amersham) and probed with anti-REG4 pAb. Protein bands were visualized using a chemiluminescent detection system (ECL; Amersham).

Akt phosphorylation. To assess the levels of phosphorylated Akt, PK-45P cells were treated with 0, 0.1, 1 or 10 nM rhREG4, with or without 100 μg/mL anti-REG4 mAb, for 6 h. Following treatment, the cells were washed with cold phosphate-buffered saline and harvested in a lysis buffer containing 50 mM HEPES (pH 7.5), 200 mM NaCl, 2.5 mM ethylenediaminetetracetic acid, 2.5 mM EGTA, 10 mM NaF, 1 mM Na_3VO_4, 0.5% Triton X-100, 0.5 mM 1,4-dithiothreitol and 0.1% protease inhibitor cocktail III (Calbiochem, San Diego, CA, USA). Samples were centrifuged and the pellet was discarded. The amount of protein present in the supernatant was measured using the Bradford method. Aliquots of 20 μg were subjected to 10% SDS-PAGE and detected by western blotting using anti-pSer473 Akt antibody (Abcam, Cambridge, MA, USA). The total amount of Akt protein was evaluated using anti-Akt antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Results

REG4 was overexpressed in PDAC cells. Through genome-wide cDNA microarray analysis, we identified dozens of genes that were overexpressed in PDAC cells.[6] Among them, we focused on *REG4* for which we confirmed its overexpression by reverse transcription-PCR in seven of the nine microdissected PDAC cell populations examined (Fig. 1A). Its mRNA levels in PDAC cells were higher than those of normal pancreas and vital organs including heart, lung, kidney and brain. Immunohistochemical analysis using pAb to REG4 in another series of PDAC tissues revealed strong signals for REG4 goblet cell-like vesicles (Fig. 1B) or at the cell surface (Fig. 1C) of cancer cells, whereas acinar cells in normal pancreas showed faint staining for REG4 (Fig. 1D) and ductal cells and islet cells showed no signal. Adult vital organs including heart, lung, kidney and brain did not show any staining either (data not shown). In addition, tissue-microarray with another series of 31 PDAC tissues spotted showed that 14 of 31 PDAC expressed high levels of REG4, and 35 out of 64 PDAC (55%) showed positive staining using anti-REG4 antibody. Well-differentiated PDAC (G1) showed positive staining for REG4 more frequently than less-differentiated PDAC (G2, G3 and G4) ($P = 0.0001$ by χ^2-test).

Serum REG4 level measured by ELISA. Secretion of REG4 was validated by measurement of the protein in the culture medium of several pancreatic cancer cell lines using anti-REG4 pAb (data not shown). Furthermore, to measure the serum REG4 level in PDAC patients, we established a sandwich ELISA method using mouse mAb 21-1, which showed the strongest affinity to human REG4 among the mAb we established, and rabbit pAb to human REG4. To determine the sensitivity of elevated REG4 as a diagnostic test, we measured serum REG4 levels of 123 healthy volunteers and defined a cut-off of 4.53 ng/mL (the mean level + 3 SD in these healthy controls). We then analyzed pre-operatively and post-operatively the serum REG4 levels of 11 patients with PDAC (Fig. 2). Pre-operative levels of REG4 in six of the 11 cases were higher than the cut-off value (cases 2, 3, 4, 5, 10 and 11), and post-operative REG4 levels in the three of these six cases (cases 3, 4 and 5) were reduced to within the normal range 3 or 4 weeks after the resection of their tumors. These results suggest that serum REG4 measured by ELISA is a promising tumor marker for PDAC. In Table 1, we summarized the clinicopathological information of these 11 patients, and there was no correlation between serum REG4 level and CA19-9 level. Nine out of the 11 patients (82%) with resectable PDAC had serum REG4 or CA19-9 elevated.

Knockdown of REG4 expression by siRNA attenuated cancer cell growth. To examine the role of REG4 overexpression in PDAC

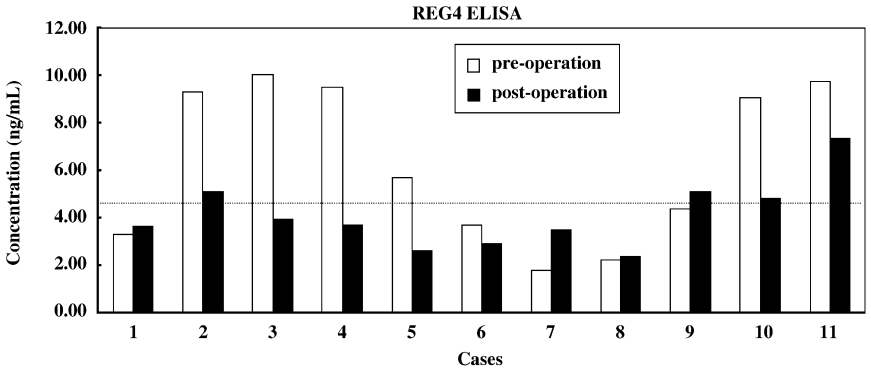

Fig. 2. Serum REG4 level in 11 patients before and after the curative resection of pancreatic ductal adenocarcinoma (PDAC). Open bar, pre-operation; closed bar, post operation. Normal range of serum REG4 was defined putatively as 4.53 ng/mL or lower (dotted line), which was determined as the mean serum level (123 healthy controls) + 3 SD. The serum levels in six of the 11 cases were higher than the cut-off value before surgical operation (cases 2, 3, 4, 5, 10 and 11). The serum REG4 levels in three cases (cases 3, 4 and 5) were reduced to within the normal range after curative resection of their tumors. The clinicopathological information and the pre-operation level of other serum markers (CA19-9 and carcinoembryonic antigen) are summarized in Table 1.

Table 1. Serum marker levels and clinicopathological summary of 11 resectable pancreatic ductal adenocarcinomas

Case	Age (years)	Location	TNM	Stage	Histology	REG4†	CA19-9‡	CEA§	Prognosis
1	56	Head	T2N1M0	III	Poorly differentiated tubular adenocarcinma	3.36	**84**	1.3	14 months dead
2	64	Head	T2N1M0	III	Moderately differentiated tubular adenocarcinoma	**9.34**	**1945**	**12.1**	9 months dead
3	69	Head	T2N0M0	I	Intraductal tubular adenocarcinoma	**10.05**	24	4.2	14 months alive
4	78	Head	T1N0M0	I	Intraductal papillary mucinous carcinoma	**9.52**	16	2.8	18 months alive
5	56	Head	T2N1M0	III	Moderately differentiated tubular adenocarcinoma	**5.72**	**311**	1.6	13 months alive
6	68	Tail	T2N0M0	I	Moderately differentiated tubular adenocarcinoma	3.73	5	1.1	8 months dead
7	70	Head	T2N1M0	III	Poorly differentiated tubular adenocarcinma	1.85	17	4.6	3 months dead
8	55	Head	T3N1M0	III	Moderately differentiated tubular adenocarcinoma	2.29	**67**	1.7	2 months alive
9	61	Head	T3N0M0	II	Poorly differentiated tubular adenocarcinma	4.43	**126**	**5.2**	5 months dead
10	74	Body	T1N1M0	III	Intraductal papillary mucinous carcinoma	**9.12**	13	4.3	9 months alive
11	78	Tail	T3N0M0	II	Moderately differentiated tubular adenocarcinoma	**9.79**	15	1.9	12 months alive

†Normal range <9.0 ng/mL; ‡normal range <36 U/mL, §normal range <5.0 ng/mL. Values above the normal range of each marker are indicated in bold.

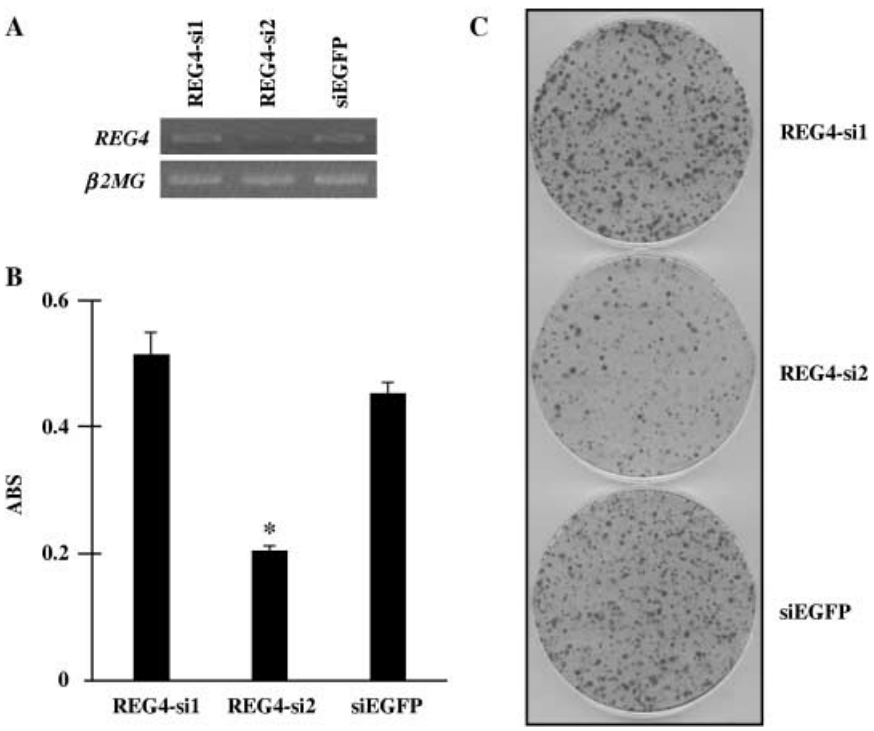

Fig. 3. Knockdown of *REG4* expression by small interfering RNA (siRNA) caused attenuation of pancreatic cancer cell growth. (A) The knockdown effect on the *REG4* transcript was validated by semiquantitative reverse transcription–polymerase chain reaction using cells transfected with each siRNA-expressing vector to REG4 (REG4-si1 and REG4-si2) and a negative control vector (siEGFP). *β2-MG* was used to quantify RNA. REG4-si2 revealed a strong knockdown effect, whereas REG4-si1 and EGFPsi did not show any effect on the level of *REG4* transcript. (B,C) Transfection of the REG4-si2 vector into SUIT-2 resulted in a significant reduction in the number of viable cells measured by MTT assay (B) and the number of colonies formed (C), compared with the cells transfected with REG4-si1 and EGFPsi vectors, which did not showed any knockdown effect on *REG4*. Columns, average of absorbance from three experiments after 7-day incubation with geneticin; bars, SD. **P* < 0.01 (Student's *t*-test) at MTT assay.

cell growth, we constructed several expression vectors designed to express siRNA specific to *REG4* and transfected them into the PDAC cell line SUIT-2, which expresses REG4 endogenously at a high level. Among the three plasmids we tested in SUIT-2 cells, REG4-si2 showed a significant knockdown effect on endogenous *REG4* transcript (Fig. 3A), and this transfection resulted in a reduction in the number of viable cells measured by MTT assay (Fig. 3B) as well as the colony formation assay (Fig. 3C), whereas

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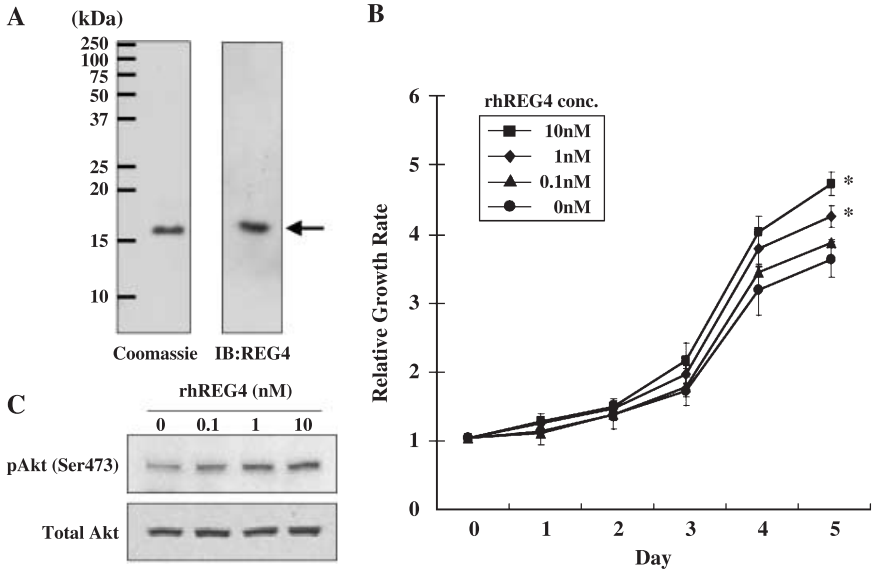

Fig. 4. Growth-promoting effect of recombinant human REG4 (rhREG4) on pancreatic ductal adenocarcinoma (PDAC) cells. (A) The bioactive rhREG4 proteins were generated using mammalian cells (FreeStyle 293F). The rhREG4 was purified and analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis, followed by Coomassie staining (left) and western blotting (right) using antibody specific to REG4. (B) PK-45P cells were incubated with 0, 0.1, 1 and 10 nM rhREG4, supplemented with 1% fetal bovine serum. The treatment with rhREG4 stimulated cell proliferation of PK-45P cells dose-dependently. Data point, average ratio of absorbance from three experiments compared with samples at day 0; bars, SD. *$P < 0.01$ (Student's *t*-test). (C) Phosphorylation of Akt (Ser473) was enhanced dose-dependently by treating PK-45P cells with 0, 0.1, 1 and 10 nM rhREG4. Phosphorylated Akt was detected by western blotting using the antibody specific to phosphorylated Akt (Ser474), and the blots were reprobed with antibody to Akt to evaluate the total level of Akt.

the transfection of other plasmids (REG4-si1 and a negative control of siEGFP) showed no knockdown effect on *REG4* expression and did not affect the cell growth of SUIT-2. In contrast, REG4-si2 did not affect the cell viability of MIAPaCa-2, which did not express REG4, excluding the possibility of an 'off-targeting' effect of REG4-si2 (data not shown). The growth suppressive effect of this siRNA-expressing vector (REG4-si2) was well correlated with the gene-silencing effects, and these data indicate a critical role for REG4 in pancreatic cancer cell survival and growth.

Treatment with rhREG4 promoted PDAC cell growth through Akt signaling. To examine the biological effects of secreted REG4 on pancreatic cancer cell growth, we generated bioactive rhREG4 protein using a mammalian system (FreeStyle 293-F) (Fig. 4A). We then carried out cell growth assays by treating PK-45P cells, which showed low expression of REG4, with several concentrations of rhREG4 (0–10 nM). Figure 4B shows that the presence of REG4 protein in culture medium stimulated cell proliferation dose-dependently, which indicates that secreted REG4 could function to promote cell proliferation extracellularly and in an autocrine/paracrine manner. One of the downstream targets of the REG family was reported to be the Akt signaling pathway.[10,12] To examine whether our rhREG4 could activate the Akt signaling pathway in PDAC cells, PK-45P cells were incubated in the presence of serial doses of rhREG4 and the phosphorylated Akt was detected by western blot analysis using an antibody specific to activated Akt with 473 serine phosphorylated. The rhREG4 treatment resulted in a significant increase in phosphorylated Akt (Fig. 4C), whereas the total expression level of Akt was not changed by the treatment with rhREG4. These data indicate that REG4 stimulates cell growth through the Akt signaling pathway in PDAC cells.

Monoclonal antibody neutralizing secreted REG4 attenuated cancer cell growth. To further evaluate the therapeutic potential of REG4 inhibition, we carried out cell growth assays by treating PDAC cells with anti-REG4 mAb. First we checked the binding affinity of several anti-REG4 mAb by immunoprecipitation using cell culture medium. Figure 5A shows that one anti-REG4 mAb (34-1) could bind endogenous REG4 protein in SUIT-2 culture medium with high affinity. Neutralization assay using PK-45P showed that the anti-REG4 mAb clone 34-1 completely offset the growth-promoting effect by rhREG4 treatment, whereas the control antibody did not show any neutralizing activity (Fig. 5B). The growth assay using SUIT-2 cells expressing endogenous REG4 at high levels showed that anti-REG4 mAb treatment inhibits SUIT-2 cell growth dose-dependently (Fig. 5C), whereas anti-REG4 mAb did not affect the cell growth of MIAPaCa-2, which did not express REG4 at all. Furthermore, we also examined the effect on Akt phosphorylation by treating PK45P cells with rhREG4 and anti-REG4 mAb. The anti-REG4 mAb treatment suppressed Akt phosphorylation in PDAC cells, which was induced by treatment with rhREG4 (Fig. 5D), indicating that anti-REG4 mAb treatment inhibits Akt signaling pathways in PDAC cells by neutralizing secreted REG4 and shutting down its autocrine/paracrine pathways. Taken together, these data suggest that anti-REG4 antibody has neutralizing activity for cell proliferation stimulated by REG4.

Discussion

In the present study, we demonstrated that approximately one-half of PDAC showed overexpression of REG4 and that elevation of serum REG4 could be detected in a subset of patients with operable PDAC, including stage I (cases 3 and 4), using our ELISA system. Because serum CA19-9 and CEA were not elevated in patients with early stage PDAC, serum REG4 is a promising candidate as a serum marker for detecting early stage PDAC. However, because several previous studies reported REG4 expression in colorectal cancer,[13] gastric cancer,[14] relapsed prostate cancer[15] and inflammatory bowl diseases,[16] the sensitivity and specificity of elevated REG4 levels in serum should be verified by a large population-based study.

In addition to the potential use of REG4 as a serum marker for PDAC diagnosis, this study demonstrated its potential as a molecular target for PDAC treatment.[17] In order to study the biological role or function of secreted REG4 in pancreatic carcinogenesis or progression, we knocked down the endogenous REG4 expression using siRNA in PDAC cell lines, and exposed cancer cells to recombinant REG4. These findings from our experiments indicate that REG4 could function as an autocrine/paracrine growth factor and mediate Akt signaling pathways, probably via an unknown receptor. How REG4 mediates the Akt signaling pathway should be investigated by further studies, as well as identification of the REG4 receptor.[18] Recent research has reported that REG4 and another REG family member, REG1, are likely to function as anti-apoptotic factors in colon and gastric cancers through the Akt pathway.[10,12] Our study validated the hypothesis that secreted REG4 activates the Akt signaling pathway, which is most likely the downstream pathway of REG

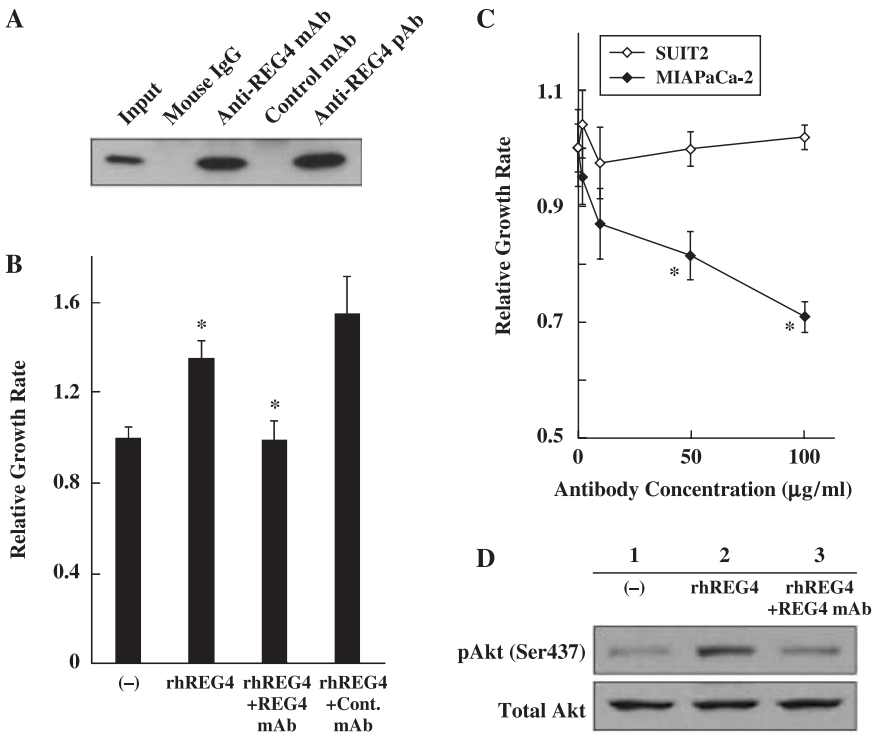

Fig. 5. Neutralizing and growth-suppressive effect of anti-REG4 monoclonal antibody. (A) Binding affinity of anti-REG4 antibodies was evaluated by immunoprecipitation using SUIT-2 culture medium, followed by western blotting using anti-REG4 polyclonal antibody. Anti-REG4 monoclonal and polyclonal antibodies could immunoprecipitate REG4 from SUIT-2 culture medium with high affinity. (B) Anti-REG4 monoclonal antibody treatment offset the growth-promoting effect of rhREG4. PK-45P was stimulated by 10 nM rhREG4 in the presence or absence of anti-REG4 monoclonal antibody. Columns, average ratios of absorbance from three experiments compared with samples grown in (–) medium; bars, SD. *$P < 0.01$ (Student's *t*-test). (C) Effects of various concentrations of anti-REG4 monoclonal antibody on the growth of SUIT-2 (REG4-positive) and MIAPaCa-2 (REG4-negative). Each cell line was incubated in the presence of various concentration of anti-REG4 monoclonal antibody. Anti-REG4 monoclonal antibody treatment suppressed SUIT-2 cell growth dose-dependently, whereas it did not affect the cell growth of MIAPaCa-2, which did not express REG4 at all. Data point, average ratios of absorbance from three experiments compared with samples grown in (–) medium; bars, SD. *$P < 0.01$ (Student's *t*-tests). (D) Anti-REG4 monoclonal antibody treatment offset the phosphorylation of Akt, which was stimulated by recombinant human REG4 (rhREG4). PK-45P cells were treated with 10 nM rhREG4 in the presence or absence of anti-REG4 monoclonal antibody. Phosphorylation of Akt was evaluated by western blotting using the antibody specific to phosphorylated Akt (Ser473), and the blots were reprobed with antibody to Akt to evaluate the total level of Akt1. 1, non-stimulated; 2, 10 nM rhREG4; 3, 10 nM rhREG4 + 100 μg/mL anti-REG4 monoclonal antibody.

family signaling associated with cancer growth and anti-apoptosis. The REG family seems to be expressed during tissue injury or the regeneration process and seems to play some an important role in tissue regeneration.[9,19] Considering that the Akt signaling pathway can be activated by REG4 and other REG family members, they may be associated with sensitivity to chemotherapy or radiation therapy of cancer, and it would be interesting to investigate the association between REG4 expression and the effect of chemo-radiation therapy *in vitro* or *in vivo*.

It is noteworthy that our monoclonal antibody specific to REG4 could neutralize secreted REG4 in the culture medium *in vitro* and treatment of these neutralizing antibodies significantly suppressed PDAC cell growth by shutting down the REG4 autocrine/paracrine pathway and blocking subsequent Akt phosphorylation. These findings implicate the feasibility of neutralizing antibody therapy targeting REG4. Bevacizumab, a humanized monoclonal antibody to vascular endothelial growth factor (VEGF), is currently approved in combination with intravenous 5-fluorouracil-containing regimens for the first-line treatment of metastatic colorectal cancer. Besides anti-angiogenesis factor antibody, antibody against circulating ligands, such as hepatocyte growth factor (HGF)[20] and interleukin-6,[21] are under review as anticancer drugs, and neutralizing-antibody therapy targeting REG4 may also provide us with a novel therapeutic strategy for PDAC and other cancers expressing REG4.

In conclusion, we here show the promising feasibility of REG4 as a serum diagnostic marker for PDAC and a molecular target for PDAC therapy, and by combining a novel strategy targeting REG4 with other screening methods or other anticancer therapeutic strategies, the prognosis of PDAC could be made more favorable than the dismal prognosis at present.

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