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(54) Title: TREATING OR PREVENTING CANCERS OVER-EXPRESSING REG4 OR KIAA0101

(57) Abstract: The invention features a method for inhibiting growth of a cancer cell by contacting the cell with a composition of an siRNA that inhibits expression of REG4 or KIAA0101. Methods of treating cancer are also within the invention. The invention also features products, including nucleic acid sequences and vectors as well as to compositions comprising them, useful in the provided methods. The invention also provides a method for inhibiting of tumor cell, for example pancreatic cancer cell, prostatic cancer cell, breast cancer cell, and bladder cancer cell, particularly pancreatic ductal adenocarcinoma (PDAC) by inhibiting REG4 gene. The present invention also relates to methods of treating or preventing PDAC in a subject comprising the step of administering to said subject a pharmaceutically effective amount of an antibody or fragment thereof that binds to a protein encoded by REG4. The present invention also relates to methods of diagnosing chemo-radiation therapeutic resistance of a cancer. The present invention also provides therapeutic agents or methods for treating cancer using the polypeptides. The polypeptides of the present invention are composed of an amino acid sequence which comprises polypeptide which comprises QKGIGEFF/SEQ ID NO: 21. The polypeptides of the present invention can be introduced into cancer cells by modifying the polypeptides with transfection agents such as poly-arginine.

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DESCRIPTION

TREATING OR PREVENTING CANCERS OVER-EXPRESSING REG4 OR KIAA0101

The present application claims the benefit of U.S. Provisional Application No. 60/838,649, filed August 18, 2006, and U.S. Provisional Application No. 60/838,749, filed August 18, 2006, the entire disclosures of each of which are hereby incorporated herein by reference for all purposes.

TECHNICAL FIELD

The present invention relates to the field of biological science, more specifically to the field of cancer research. In particular, the present invention also relates to methods of treating and preventing cancer, for example pancreatic cancer, prostatic cancer, breast cancer, and bladder cancer. In particular, the present invention relates a composition comprising a nucleic acid capable of inhibiting expression of the gene encoding REG4 and KIAA0101. In some embodiments, the compound is a small interfering RNA (siRNA) corresponding to a subsequence from these genes.

Alternatively, the present invention also relates to methods of treating or preventing pancreatic cancer, especially pancreatic ductal adenocarcinoma (PDAC), in a subject comprising the step of administering to said subject a pharmaceutically effective amount of an antibody or fragment thereof that binds to a protein encoded by REG4. Moreover, the present invention relates a composition comprising a peptide of inhibiting the interaction of KIAA0101 with PCNA. In some embodiments, the compound is a cell-permeable dominant-negative peptides have conserved PCNA-binding motif (PIP box).

BACKGROUND ART

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the western world and shows the worst mortality among the common malignancies, with a 5-year survival rate of only 4% (DiMagno EP, *et al.* Gastroenterology 1999; 117: 1464-84., Wray CJ, *et al.* Gastroenterology 2005; 128: 1626-41.). In 2006, it is estimated that approximately 33,730 new cases are diagnosed to have pancreatic cancer in the United States and 32,300 of them are likely to die of the disease (Jemal A, *et al.* Cancer statistics, 2006. CA Cancer J Clin 2006; 56: 106-130.). Since the majority of PDAC patients are diagnosed at their advanced stage, no effective therapy to cure the disease is available at

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present. Several approaches in a combination of surgery with chemotherapy, including 5-FU or gemcitabine, with or without radiation, can improve patients' quality of life (DiMagno EP, *et al.* Gastroenterology 1999; 117: 1464-84., Wray CJ, *et al.* Gastroenterology 2005; 128: 1626-41.), but those treatments have a very limited effect on long-term survival of PDAC patients due to its extremely aggressive and chemo-resistant nature.

The very poor prognosis of PDAC arises from several reasons that include the difficulty of detection of PDACs at an early stage (DiMagno EP, *et al.* Gastroenterology 1999; 117: 1464-84., Wray CJ, *et al.* Gastroenterology 2005; 128: 1626-41.). Despite improvements in diagnostic imaging techniques such as endoscopic ultrasound ("EUS") or magnetic resonance cholangiopancreatography ("MRCP") (DiMagno EP, *et al.* Gastroenterology 1999; 117: 1464-84., Wray CJ, *et al.* Gastroenterology 2005; 128: 1626-41.), 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 PSA (prostate-specific antigen) for prostate cancer, could facilitate detection of PDACs at an early stage and can be applied for mass-screening of PDACs. Surgical resection of early-staged PDACs can offer the relatively favorable prognosis as 50-60% of five-year survival (Wray CJ, *et al.* Gastroenterology 2005; 128: 1626-41.). Hence, considering biological aggressiveness and resistance to chemotherapy of PDACs, one of the most realistic strategies to improve the prognosis of this fatal disease is to screen PDACs at an early stage by a non-invasive serological test.

Currently, CA19-9 is the only commercially available serological marker for PDACs, but it is far from an ideal tumor marker, 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, and (iii) it is usually within a normal range in patients at an early stage (Sawabu N, *et al.* Pancreas 2004; 28: 263-7., Pleskow DK, *et al.* Ann Intern Med 1989; 110: 704-9.). Hence, establishment of a screening strategy thorough development of a novel tumor maker that is more specific and more sensitive to PDACs is urgently required.

REG4 has been reported to be a new member of the REG family (Hartupee JC, *et al.* Biochim Biophys Acta 2001; 1518: 287-93.), and as a tumor marker of PDAC. The molecules belonging to the REG (regenerating islet-derived) family are secreted proteins playing a role in tissue regeneration and inflammation in digestive organs (Hartupee JC, *et al.*

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Biochim Biophys Acta 2001; 1518: 287-93., Watanabe T, *et al.* J Biol Chem 1990; 265: 7432-9., Uno M, *et al.* Adv Exp Med Biol 1992; 321: 61-6.). The expression levels of the members were reported to be up-regulated in several gastrointestinal cancers and to function as a trophic or anti-apoptotic factor in cancers (Unno M, *et al.* Adv Exp Med Biol 1992; 321: 61-6., Sekikawa A, *et al.* Gastroenterology 2005; 128: 642-53.).

cDNA microarray technologies have enabled practitioners to obtain comprehensive profiles of gene expression in normal and malignant cells, and compare the gene expression in malignant and corresponding normal cells (Okabe *et al.*, (2001) Cancer Res 61:2129-37; Kitahara *et al.*, (2001) Cancer Res 61: 3544-9; Lin *et al.*, (2002) Oncogene 21:4120-8; Hasegawa *et al.*, (2002) Cancer Res 62:7012-7). This approach enables the disclosure of the complex nature of cancer cells, and helps to understand the mechanism of carcinogenesis. Identification of genes that are deregulated in tumors will lead to more precise and accurate diagnosis of individual cancers, and to develop novel therapeutic targets (Bienz and Clevers, (2000) Cell 103:311-20).

For example, recent years, a new approach of cancer therapy using gene-specific siRNA was attempted in clinical trials (Bumcrot D *et al.*, Nat Chem Biol 2006 Dec, 2(12): 711-9). RNAi seems to have already earned a place among the major technology platforms (Putral LN *et al.*, Drug News Perspect 2006 Jul-Aug, 19(6): 317-24; Frantz S, Nat Rev Drug Discov 2006 Jul, 5(7): 528-9; Dykxhoorn DM *et al.*, Gene Ther 2006 Mar, 13(6): 541-52). Nevertheless, there are several challenges that need to be faced before RNAi can be applied in clinical use. These challenges include overcoming poor stability of RNA *in vivo* (Hall AH *et al.*, Nucleic Acids Res 2004 Nov 15, 32(20): 5991-6000, Print 2004; Amarzguioui M *et al.*, Nucleic Acids Res 2003 Jan 15, 31(2): 589-95), reducing the toxicity as an agent (Frantz S, Nat Rev Drug Discov 2006 Jul, 5(7): 528-9) and selecting the suitable mode of delivery, the precise sequence of the siRNA or shRNA used, and cell type specificity. It is well-known fact that there are possible toxicities related to non-specific silencing because of partial homology, or induction of the interferon response by inducing double-stranded molecules (Judge AD *et al.*, Nat Biotechnol 2005 Apr, 23(4): 457-62, Epub 2005 Mar 20; Jackson AL & Linsley PS, Trends Genet 2004 Nov, 20(11): 521-4). So, double-stranded molecules targeting cancer-specific genes must be improved to be devoid of adverse effects.

Earlier the present inventors performed detailed and accurate expression profile analysis of pancreatic cancers using a genome-wide cDNA microarray consisting of

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approximately 27,000 genes, in combination with laser microdissection to purify cancer cell population (Nakamura et al., (2004) *Oncogene*, 23: 2385-400). Among the genes the present inventors identified as being trans-activated in pancreatic cancer cells, the present inventors here focused on KIAA0101 as a molecular target for cancer therapy (WO2004/031412).

5 PCNA (proliferating cell nuclear antigen) is essential for DNA replication and DNA repair as well as influencing cell cycle progression through interacting with several cell cycle proteins (Prelich et al., (1987) *Nature*, 326: 471-5; Wyman and Botchan (1995) *Curr Biol*, 5: 334-7; Warbrick et al., (2000) *Bioessays*, 22: 997-1006). The crystal structure showed that PCNA is a ring-shaped homotrimeric protein and functions as a clamping
10 platform necessary to recruit to DNA proteins involved in DNA synthesis or metabolism, such as DNA polymerases, DNA ligase, and others (Krishna et al., (1994) *Cell*, 79: 1233-43). PCNA interacts with numerous DNA replication/repair enzymes, and several proteins have its conserved PCNA-binding motif (PIP box, QXXL/I/MXXF/Y) through which they interact with PCNA (Jonsson et al., (1998) *EMBO J*, 17: 2412-25). Overexpression of PCNA is a
15 hallmark of cell proliferation and in the clinic PCNA serves as a general proliferative marker, especially in the prognosis of tumor development as well as Ki67/ MIB-1 (Haitel et al., (1997) *Am J Clin Pathol*, 107: 229-35).

KIAA0101 was previously identified as p15PAF (PCNA-associated factor) to bind with PCNA protein by yeast two-hybrid screening (Yu et al., (2001) *Oncogene* 20: 484-9) and
20 it has the conserved PIP box through which it can interact with PCNA. However, its function remains unknown and how KIAA0101-PCNA interaction can involve cell proliferation or cancer progression is still a puzzle (Yu et al., (2001) *Oncogene*, 20: 484-9; Simpson et al., (2006) *Exp Cell Res*. 312: 73-85).

SUMMARY OF THE INVENTION

25 The present inventors here report over-expression of REG4, a new member of the REG family, and/or KIAA0101 in cancer cells (*e.g.*, in PDAC cells) on the basis of the genome-wide cDNA microarray analysis as well as RT-PCR and immunohistochemical analysis. Furthermore, the present inventors found that knockdown of the endogenous REG4 and/or KIAA0101 expression in cancer (*e.g.*, PDAC) cell lines with an siRNA caused
30 drastic decrease of cell viability. Concordantly, addition of recombinant REG4 and/or KIAA0101 to the culture medium enhanced growth of cancer (*e.g.*, PDAC cell lines in a dose-dependent manner. A monoclonal antibody against REG4 and/or KIAA0101 neutralizes the

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growth-promoting effects and attenuated significantly the growth of cancer (*e.g.*, PDAC cells). These findings implicate that REG4 and KIAA0101 are promising tumor markers to screen early-staged cancers, including PDAC, and also that neutralization of REG4 and/or KIAA0101, for example, by the use of an antibody, an inhibitory polypeptide or an inhibitory polynucleotide (*e.g.*, siRNA) offers an effective prophylactic or therapeutic treatment against cancers mediated by aberrant (*i.e.*, abnormally high) REG4 and/or KIAA0101 over-expression and/or intracellular signaling, including PDACs.

An objective of the present invention is to provide compounds useful in the treatment and/or prevention of cancers mediated by aberrant (*i.e.*, abnormally high) REG4 and/or KIAA0101 over-expression and/or intracellular signalling. Alternatively, an objective of the present invention is to provide pharmaceutical compositions and methods for either or both the treatment and prevention of such cancers, for example, pancreatic cancer, prostate cancer, breast cancer or bladder cancer using the compounds.

The present inventors confirm that suppression of REG4 or KIAA0101 expression, for example using an siRNA, can achieve the inhibition of cancer proliferation. Accordingly, it is an objective of the present invention is to provide method for treating or preventing pancreatic cancer in a subject comprising administering to said subject a composition comprising a small interfering RNA (siRNA) that inhibits expression of REG4. In a further embodiment, the invention provides methods for treating or preventing cancers mediated by aberrant (*i.e.*, abnormally high) KIAA0101 over-expression and/or intracellular signaling, for example, pancreatic cancer, prostate cancer, breast cancer or bladder cancer in a subject comprising administering to said subject a composition comprising an siRNA that inhibits expression of KIAA0101.

The present invention also provides a pharmaceutical composition for treating or preventing pancreatic cancer comprising a pharmaceutically effective amount of a small interfering RNA (siRNA) that inhibits expression of REG4 as an active ingredient, and a pharmaceutically acceptable carrier. The present invention further provides a pharmaceutical composition for treating or preventing cancers mediated by aberrant (*i.e.*, abnormally high) KIAA0101 over-expression and/or intracellular signaling, for example, pancreatic cancer, prostate cancer, breast cancer or bladder cancer comprising a pharmaceutically effective amount of an siRNA that inhibits expression of KIAA0101 as an active ingredient, and a pharmaceutically acceptable carrier.

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The present invention further provides use of a small interfering RNA (siRNA) that inhibits expression of REG4 for manufacturing a pharmaceutical composition for treating or preventing pancreatic cancer, and use of a small interfering RNA (siRNA) that inhibits expression of KIAA0101 for manufacturing a pharmaceutical composition for treating or preventing cancers mediated by aberrant (*i.e.*, abnormally high) KIAA0101 over-expression and/or intracellular signaling, for example, pancreatic cancer, prostate cancer, breast cancer or bladder cancer. In some embodiments, a preferable siRNA comprises the nucleotide sequence of SEQ ID NO: 5 or SEQ ID NO: 32 as the target sequence.

The present invention relates to a double-stranded molecule comprising a sense strand and an antisense strand, wherein the sense strand comprises a ribonucleotide sequence corresponding to a target sequence of SEQ ID NO: 5 or SEQ ID NO: 32, and wherein the antisense strand comprises a ribonucleotide sequence which is complementary to said sense strand, wherein said sense strand and said antisense strand hybridize to each other to form said double-stranded molecule, and wherein said double-stranded molecule, when introduced into a cell expressing the REG4 gene or the KIAA0101 gene, inhibits expression of said gene.

In addition, it is confirmed that REG4 functions as an autocrine or paracrine growth factor and mediate Akt signaling pathways. Furthermore, the present inventors find that an anti-REG4 antibody neutralizes the cell proliferative activity of the REG4 to attenuate the growth of pancreatic cancer cells.

Accordingly, it is an objective of the present invention is to provide a method for treating or preventing pancreatic cancer in a subject comprising administering to said subject an anti-REG4 antibody or fragment thereof that neutralizes REG4 activity. The present invention also provides a pharmaceutical composition for treating or preventing pancreatic cancer, said composition comprising a pharmaceutically effective amount of an anti-REG4 antibody or fragment thereof that neutralizes REG4 activity as an active ingredient, and a pharmaceutically acceptable carrier. The present invention further provides use of an anti-REG4 antibody or fragment thereof that neutralizes REG4 activity for manufacturing a pharmaceutical composition for treating or preventing pancreatic cancer. In some embodiments, preferable anti-REG4 antibody is monoclonal antibody. In some embodiments, anti-REG4 antibody comprises a VH and VL chain, each VH and VL chain comprising CDR amino acid sequences designated CDR1, CDR2 and CDR3 separated by framework amino acid sequences, the amino acid sequence of each CDR in each VH and VL chain is:

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VH CDR1 : SYWMH (SEQ ID NO: 20),
VH CDR2 : NIYPGSGSTNYD (SEQ ID NO: 21),
VH CDR3 : GGLWLRVDY (SEQ ID NO: 22),
VL CDR1 : SASSSVSYM H (SEQ ID NO: 23),
VL CDR2 : DTSKLAS (SEQ ID NO: 24), and
VL CDR3 : QQWSSNPF (SEQ ID NO: 25).

The present invention also relates to methods for treatment and/or prevention of pancreatic cancer comprising the step of administering an antibody comprises a VH and VL chain, each VH and VL chain comprising CDR amino acid sequences designated CDR1, CDR2 and CDR3 separated by framework amino acid sequences, the amino acid sequence of each CDR in each VH and VL chain is:

VH CDR1 : SYWMH (SEQ ID NO: 20),
VH CDR2 : NIYPGSGSTNYD (SEQ ID NO: 21),
VH CDR3 : GGLWLRVDY (SEQ ID NO: 22),
VL CDR1 : SASSSVSYM H (SEQ ID NO: 23),
VL CDR2 : DTSKLAS (SEQ ID NO: 24), and
VL CDR3 : QQWSSNPF (SEQ ID NO: 25).

In addition, the present invention provides inhibitory polypeptides that contain QKGIGEFF (SEQ ID NO: 46). In some preferred embodiments, the amino acid sequence is VRPTPKWQKGIGEFFRLSPK (SEQ ID NO. 44) or TPKWQKGIGEFFRLSP (SEQ ID NO. 45). The present invention further provides pharmaceuticals or methods using these inhibitory polypeptides for prevention and/or treatment of cancer.

The present invention also relates to methods for treatment and/or prevention of cancer comprising the step of administering an inhibitory polypeptide that contains QKGIGEFF (SEQ ID NO: 46), for example an inhibitory polypeptide having at least a fragment of the amino acid sequence VRPTPKWQKGIGEFFRLSPK (SEQ ID NO: 44); TPKWQKGIGEFFRLSP (SEQ ID NO. 45); or a polynucleotide encoding the same. Furthermore, the present invention relates to the use of polypeptides of the invention; or the use of nucleotides encoding the same, in manufacturing pharmaceutical formulations for the treatment and/or prevention of cancer.

These and other objects and features of the invention will become more fully apparent when the following detailed description is read in conjunction with the

accompanying figures and examples. However, it is to be understood that both the foregoing summary of the invention and the following detailed description are a preferred embodiment, and not restrictive of the invention or other alternate embodiments of the invention.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 REG4 mRNA and protein expression levels in PDAC cells. (A) RT-PCR analysis of REG4 and TUBA, as a quantitative control, in the microdissected PDAC cells (lane1-9) comparing 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, C, D) In immunohistochemical study using anti-REG4 antibody, intense staining was observed in PDAC cells. Positive staining of REG4 was observed as cytoplasmic granules (B), suggesting secretion of REG4, 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).

Fig. 2 Knockdown of REG4 expression by siRNA caused attenuation of pancreatic cancer cell growth. (A) Knockdown effect on REG4 transcript was validated by semi-quantitative RT-PCR using cells transfected with an siRNA expressing vector to REG4 (REG4-si2) and a negative control vector (siEGFP). β 2-MG was used to quantify RNAs. REG4-si2 revealed strong knockdown effect, while EGFPsi did not show any effect on the level of REG4 transcript. (B, C) Transfection with a REG4-si2 vector into SUTT-2 resulted in drastic reduction of the numbers of viable cells measured by MTT assay (B) and the number of colony formation (C), compared with the cells transfected with EGFPsi vectors which did not showed any knockdown effect on REG4. Columns, average of absorbance from three experiments after a 7 day incubation with Geneticin; bars, SD. * $p < 0.01$ (Student's *t*-test) at MTT assay (B).

Fig. 3 Growth-promoting effect of rhREG4 on PDAC cells. (A) The bioactive rhREG4 proteins were generated by mammalian cells (FreeStyleTM 293). The rhREG4 was purified and analyzed by SDS-PAGE, followed by Coomassie staining (left) and Western blot (right) using specific antibody to REG4. (B) PK-45P cells were incubated with 0, 0.1, 1 and 10nM rhREG4, supplied with 1%FBS. The treatment of rhREG4 stimulated cell proliferation of PK-45P cells dose-dependently. Data point, average ratios of absorbance from three experiments compared with samples day 0; bars, SD. * $p < 0.01$ (Student's *t* test). (C) Phosphorylation of Akt (Ser473) was enhanced dose-dependently by treating PK-45P

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cells with 0, 0.1, 1 and 10nM rhREG4. Phosphorylated Akt was detected by Western blot using the antibody specific to phosphorylated Akt (Ser473), and the blots were reprobed with antibody to Akt to evaluate the total level of Akt.

Fig. 4 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 blot using anti-REG4 pAb. Anti-REG4 mAb and pAb immunoprecipitated REG4 from SUIT-2 culture medium with high affinity. (B) Anti-REG4 mAb treatment offset the growth-promoting effect of rhREG4. PK-45P was stimulated by 10nM rhREG4 in the presence or absence of anti-REG4 mAb. Columns, average ratios of absorbance from three experiments compared with samples grown in (-) medium; bars, SD. * $p < 0.01$ (Student's t tests). (C) Effects of various concentration of anti-REG4 mAb 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 mAb. Anti-REG4 mAb treatment suppressed SUIT-2 cell growth dose-dependently while it did not affect the cell growth of MIAPaCa-2 that 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 mAb treatment offset the phosphorylation of Akt that was stimulated by rhREG4. PK-45P cells were treated with 10nM rhREG4 in presence or absence of anti-REG4 mAb. Phosphorylation of Akt was evaluated by Western blot using the antibody specific to phosphorylated Akt (Ser473), and the blots were reprobed with antibody to Akt to evaluate the total level of Akt. 1, non-stimulated; 2, 10nM rhREG4; 3, 10nM rhREG4 + anti-REG4 mAb.

Fig. 5 Anti-REG4 antibody treatment suppressed pancreatic cancer cell growth *in vivo*. (A) Tumor volumes which were inoculated into nude mice were evaluated during the treatment of anti-REG4 antibody (34-1 mAb; n=8) with two times per a week (300 μ g/mouse i.p.) or control antibody (normal mouse IgG; n=9). The treatment of 34-1 mAb (shown in solid round marker and bold line) induced significant reduction of tumor volumes comparing to control IgG ($P=0.0598$). (B) Tumors were weighted at 30 days after first treatment of anti-REG4 antibody (34-1 mAb) or control antibody. The treatment of 34-1 mAb (shown in black box) induced significant reduction of tumor weights comparing to control antibody ($P=0.0489$).

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Fig. 6 REG4 over-expression contributed to γ -ray resistance of pancreatic cancer. (A) pCAGGSnHC-REG4-HA was transfected to pancreatic cancer cell line PK-45P that did not express REG4. After the selection of Geneticin resistant cells, expression of recombinant REG4 at cell line was checked by western blotting assay. (B) After 48 hours pre-incubation, REG4-expressing clones (C1-6, C2-6 and C10) or control clones (M1, M3 and M6) were γ -irradiated at 1, 5, 10, or 30 Gy by using a ^{60}Co source. After 48 hours, viable cells were measured by using cell-counting, and relative ratio of absorbance-(each irradiation)/absorbance-(no-irradiation) was evaluated. (C) After 0, 1, or 5 Gy γ -irradiation, γ ray-induced apoptosis was evaluated by detecting sub-G1 fraction using flow cytometer.

Fig. 7 REG4 over-expression contributed to gemcitabine-resistance of pancreatic cancer. (A) After 48 hours pre-incubation, REG4-expressing clones (C1-6, C2-6, C10) or control clones (M1, M3, M6) were treated with 0.1-100,000 nM gemcitabine for 48 hours. After incubation, viable cells were measured by using Cell-counting, and relative ratio of absorbance-(each treatment)/absorbance-(no treatment control) was evaluated. (B) After treating 10nM or 50nM gemcitabine for 48 hours, apoptosis was evaluated by detecting sub-G1 fraction using flow cytometer.

Fig. 8 The immunostaining of REG4 in the pancreatic adenocarcinoma specimens undergoing neo-CRT. (A), (B) The pancreatic adenocarcinoma specimens responding to neo-adjuvant chemo-radiation therapy (neo-CRT) showed low or no expression of REG4. (C), (D) The pancreatic adenocarcinoma specimens non-responding to neo-CRT showed strong expression of REG4.

Fig. 9 The results of over-expression of KIAA0101 in pancreatic cancer cells. (A) Semi-quantitative RT-PCR validated that KIAA0101 expression was up-regulated in the microdissected pancreatic cancer cells compared with normal pancreatic duct cells which were also microdissected and normal pancreatic tissue. Expression of TUBA served as the quantitative control. (B) Northern blot analysis showed the strong expression of KIAA0101 in pancreatic cancer cell lines (lanes 1-6), while no expression was observed in vital organs including heart, lung, liver, kidney, and brain (lanes 7-11). (C) Immunohistochemical study using anti-KIAA0101 antibody. Intense staining was observed in the nuclei of pancreatic cancer cells (arrowhead x400), while acinar cells and normal ductal epithelium in normal pancreatic tissue showed no staining.

Fig. 10 The result of effect of KIAA0101 knockdown by siRNAs on growth of pancreatic cancer cells. (A) Two siRNA expression vectors specific to KIAA0101 transcript (#759si) and an EGFP siRNA expression vector (EGFPsi) as a negative control were transfected into KLM-1 cells. Knockdown effect on KIAA0101 transcript was validated by RT-PCR, with β 2MG expression as a quantitative control. Transfection with #759si showed strong knockdown effect, while EGFPsi did not show any effect on the level of KIAA0101 transcript. (B) Transfection with #759si vector resulted in drastic reduction of the numbers of viable cells measured by the number of colony formation, compared with the cells transfected with siRNA expression vector in which did not showed their knockdown effect on KIAA0101. (C) Transfection with #759si vector resulted in drastic reduction of the numbers of viable cells measured by MTT assay. ABS on Y-axis at MTT assay means absorbance at 490 nm, and at 630 nm as reference, measured with a microplate reader.

Fig. 11 The result of exogenous over-expression of KIAA0101 promoted cancer cell growth and transformed NIH3T3. (A) Western blot analysis of six PK-45P derivatives cells (clones 1-6) expressing exogenous KIAA0101 constitutively and those transfected with mock vector (Mock 1-4). Exogenous introduction of KIAA0101 expression was validated with anti-HA tag antibody. ACTB served as a loading control. (B) The growth measurement by MTT assay demonstrates that the six KIAA0101 clones (1-6, solid lines) grew significantly more rapidly than the two mock clones (1-4, dash lines). X-, and Y-axis represent day point after seeding and relative growth rate that was calculated in absorbance of the diameter by comparison with the absorbance value of day 1 as a control. Each average is plotted with error bars representing standard error. These experiences were in triplicate altogether. (C) Three KIAA0101-overexpressed clones and three mock clones were established from NIH3T3 that did not express endogenous KIAA0101 mouse homologue. Three KIAA0101-overexpressed NIH3T3 clones (1-3) and mock NIH3T3 clones were inoculated in the right and left flank of 8-week nude mice, respectively. After four weeks, only KIAA0101-overexpressed NIH3T3 cells formed the mass at the right flank of nude mice. (D) Each of the tumors was immunostained by anti-KIAA0101 antibody, showing the exogenous KIAA0101 expression.

Fig. 12 KIAA0101 protein was associated with several DNA replication proteins. (A) Immunoprecipitated fractions separated on SDS-PAGE gels followed by silver staining showed that several proteins were immunoprecipitated with KIAA0101 proteins from cancer

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cell lysate, compared with results from a control sample. Each differential band was analyzed by a MALDI-TOF system after in-gel trypsin digestion; they were identified as PCNA, POLD1 (polymerase δ p125 subunit), and FEN1 (flap endonuclease-1). (B) These interactions were confirmed by immunoprecipitation experiment. All of these proteins are involved with DNA replication and POLD1 and FEN1 also bind to PCNA as well as KIAA0101.

Fig. 13 The inhibition of the interaction between KIAA0101 and PCNA by cell-permeable dominant-negative peptides. (A) Two dominant-negative peptides (PIP20, PIP16) containing PIP box ({q}KG{i}GE{ff}/SEQ ID NO: 21 shown in parentheses) and its mutant peptides (PIP20mt, PIP16mt) with the conserved PIP box residues replaced with alanines ({a}KG{a}GE{aa}/SEQ ID NO: 37 shown in parentheses) were designed and conjugated them with arginine (R)-repeat to facilitate cell permeability. (B) In vitro study, immunoprecipitation validated the inhibition of the interaction between PCNA and KIAA0101 by PIP20 treatment, but PIP20mt and scramble peptides did not affect the interaction between PCNA and KIAA0101. (C) PIP20 treatment suppressed cell growth of KIAA0101 with KIAA0101 expression dose-dependently, while PIP20mut and scramble peptide did not. On the other hand, PIP20 did not affect the growth of mouse normal cell line NIH3T3 cells that did not express the homologue of human KIAA0101. (D) Short PIP peptides (PIP16 and PIP16mt) were designed by delete four residues of N- and C-terminus with PIP box motif maintained, and PIP16 treatment suppressed cancer cell growth strongly, however, PIP16 also affected NIH3T3 growth.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Small interfering RNA:

The present invention based in part on the surprising discovery that inhibiting expression of REG4 and/or KIAA0101 is effective in inhibiting the cellular growth of various cancer cells, including those involved in pancreatic cancer, prostate cancer, breast cancer and bladder cancer. In particular, it has been surprisingly discovered that PDAC can be prevented or inhibited by inhibiting REG4 gene. The inventions described in this application are based in part on these discoveries.

The invention provides methods for inhibiting cell growth. Among the methods provided are those comprising contacting a cell with a composition comprising a small interfering RNA (siRNA) that inhibits expression (*i.e.*, transcription or translation) of REG4

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or KIAA0101. The invention also provides methods for inhibiting tumor cell growth in a subject. Such methods include administering to a subject a composition comprising a small interfering RNA (siRNA) that hybridizes specifically to a sequence from REG4 or KIAA0101. Another aspect of the invention provides methods for inhibiting the expression of the REG4
5 gene or KIAA0101 gene in a cell of a biological sample.

Expression of the gene may be inhibited by introduction of a double stranded ribonucleic acid (RNA) molecule into the cell in an amount sufficient to inhibit expression of the REG4 gene or KIAA0101 gene. Another aspect of the invention relates to products including nucleic acid sequences and vectors as well as to compositions comprising them,
10 useful, for example, in the provided methods. Among the products provided are the siRNA molecules having the property to inhibit expression of the REG4 gene or KIAA0101 gene when introduced into a cell expressing said gene. Among such molecules are those that comprise a sense strand and an antisense strand, wherein the sense strand comprises a ribonucleotide sequence corresponding to a REG4 or KIAA0101 target sequence, and
15 wherein the antisense strand comprises a ribonucleotide sequence which is complementary to said sense strand. The sense and the antisense strands of the molecule hybridize to each other to form a double-stranded molecule.

As used herein, the term "organism" refers to any living entity comprised of at least one cell. A living organism can be as simple as, for example, a single eukaryotic cell or as
20 complex as a mammal, including a human being.

As used herein, the term "biological sample" refers to a whole organism or a subset of its tissues, cells or component parts (*e.g.* bodily fluids, including but not limited to blood, mucus, lymphatic fluid, synovial fluid, cerebrospinal fluid, saliva, amniotic fluid, amniotic cord blood, urine, vaginal fluid and semen). "Biological sample" further refers to a
25 homogenate, lysate, extract, cell culture or tissue culture prepared from a whole organism or a subset of its cells, tissues or component parts, or a fraction or portion thereof. Lastly, "biological sample" refers to a medium, such as a nutrient broth or gel in which an organism has been propagated, which contains cellular components, such as proteins or nucleic acid molecules.

30 The invention features methods of inhibiting cell growth. Cell growth can be inhibited by contacting a cell with a composition of a small interfering RNA (siRNA) of REG4 or KIAA0101. The cell can be further contacted with a transfection-enhancing agent. The cell can be provided *in vitro*, *in vivo* or *ex vivo*. The subject can be a mammal, *e.g.*, a

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human, non-human primate, mouse, rat, dog, cat, horse, or cow. The cell can be a pancreatic ductal cell. Alternatively, the cell can be a tumor cell (*i.e.*, cancer cell), for example, a carcinoma cell or an adenocarcinoma cell. For example, the cell can be a pancreatic cancer cell, especially pancreatic ductal adenocarcinoma cell, prostatic cancer cell, breast cancer cell, or a bladder cancer cell. By inhibiting cell growth is meant that the treated cell proliferates at a lower rate or has decreased viability than an untreated cell. Cell growth is measured by proliferation assays known in the art.

The term "polynucleotide" and "oligonucleotide" are used interchangeably herein unless otherwise specifically indicated and are referred to by their commonly accepted single-letter codes. The terms apply to nucleic acid (nucleotide) polymers in which one or more nucleic acids are linked by ester bonding. The polynucleotide or oligonucleotide may be composed of DNA, RNA or a combination thereof.

As used herein, the term "double-stranded molecule" refers to a nucleic acid molecule that inhibits expression of a target gene including, for example, short interfering RNA (siRNA; *e.g.*, double-stranded ribonucleic acid (dsRNA) or small hairpin RNA (shRNA)) and short interfering DNA/RNA (siD/R-NA; *e.g.* double-stranded chimera of DNA and RNA (dsD/R-NA) or small hairpin chimera of DNA and RNA (shD/R-NA)).

By the term "siRNA" is meant a double stranded RNA molecule which prevents translation of a target mRNA. Standard techniques of introducing siRNA into the cell are used, including those in which DNA is a template from which RNA is transcribed. The siRNA includes a sense REG4 or KIAA0101 nucleic acid sequence, an antisense REG4 or KIAA0101 nucleic acid sequence or both. The siRNA may comprise two complementary molecules or may be constructed such that a single transcript has both the sense and complementary antisense sequences from the target gene, *e.g.*, a hairpin, which, in some embodiments, leads to production of micro RNA (miRNA). The siRNA may either be a dsRNA or shRNA.

As used herein, the term "dsRNA" refers to a construct of two RNA molecules comprising complementary sequences to one another and that have annealed together via the complementary sequences to form a double-stranded RNA molecule. The nucleotide sequence of two strands may comprise not only the "sense" or "antisense" RNAs selected from a protein coding sequence of target gene sequence, but also RNA molecule having a nucleotide sequence selected from non-coding region of the target gene.

The term "shRNA", as used herein, refers to an siRNA having a stem-loop structure,

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comprising a first and second regions complementary to one another, *i.e.*, sense and antisense strands. The degree of complementarity and orientation of the regions being sufficient such that base pairing occurs between the regions, the first and second regions being joined by a loop region, the loop resulting from a lack of base pairing between nucleotides (or nucleotide
5 analogs) within the loop region. The loop region of an shRNA is a single-stranded region intervening between the sense and antisense strands and may also be referred to as “intervening single-strand”.

As use herein, the term “siD/R-NA” refers to a double-stranded polynucleotide molecule which is composed of both RNA and DNA, and includes hybrids and chimeras of
10 RNA and DNA and prevents translation of a target mRNA. Herein, a hybrid indicates a molecule wherein a polynucleotide composed of DNA and a polynucleotide composed of RNA hybridize to each other to form the double-stranded molecule; whereas a chimera indicates that one or both of the strands composing the double stranded molecule may contain RNA and DNA. Standard techniques of introducing siD/R-NA into the cell are used. The
15 siD/R-NA includes a CX sense nucleic acid sequence (also referred to as “sense strand”), a CX antisense nucleic acid sequence (also referred to as “antisense strand”) or both. The siD/R-NA may be constructed such that a single transcript has both the sense and complementary antisense nucleic acid sequences from the target gene, *e.g.*, a hairpin. The siD/R-NA may either be a dsD/R-NA or shD/R-NA.

As used herein, the term “dsD/R-NA” refers to a construct of two molecules
20 comprising complementary sequences to one another and that have annealed together via the complementary sequences to form a double-stranded polynucleotide molecule. The nucleotide sequence of two strands may comprise not only the “sense” or “antisense” polynucleotides sequence selected from a protein coding sequence of target gene sequence,
25 but also polynucleotide having a nucleotide sequence selected from non-coding region of the target gene. One or both of the two molecules constructing the dsD/R-NA are composed of both RNA and DNA (chimeric molecule), or alternatively, one of the molecules is composed of RNA and the other is composed of DNA (hybrid double-strand).

The term “shD/R-NA”, as used herein, refers to an siD/R-NA having a stem-loop
30 structure, comprising a first and second regions complementary to one another, *i.e.*, sense and antisense strands. The degree of complementarity and orientation of the regions being sufficient such that base pairing occurs between the regions, the first and second regions being joined by a loop region, the loop resulting from a lack of base pairing between nucleotides (or

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nucleotide analogs) within the loop region. The loop region of an shD/R-NA is a single-stranded region intervening between the sense and antisense strands and may also be referred to as "intervening single-strand".

The method is used to alter gene expression in a cell in which expression of REG4 or KIAA0101 is aberrantly up-regulated, *e.g.*, as a result of malignant transformation of the cells. Binding of the siRNA to a REG4 or KIAA0101 transcript in the target cell results in a reduction in REG4 or KIAA0101 production by the cell. The length of the oligonucleotide is at least about 10 nucleotides and may be as long as the naturally-occurring REG4 or KIAA0101 transcript. Preferably, the oligonucleotide is less than about 75, about 50, or about 25 nucleotides in length. Most preferably, the oligonucleotide is about 19 to about 25 nucleotides in length. Examples of the siRNA oligonucleotides of REG4 or KIAA0101 which inhibit REG4 or KIAA0101 expression in mammalian cells include oligonucleotides containing target sequences, for example, nucleotide of SEQ ID NO: 5 or SEQ ID NO: 32, respectively.

Methods for designing double stranded RNA having the ability to inhibit gene expression in a target cell are known. (*See* for example, US Patent No. 6,506,559, herein incorporated by reference in its entirety). For example, a computer program for designing siRNAs is available from the Ambion website (www.ambion.com/techlib/misc/siRNA_finder.html). The computer program available from Ambion, Inc. selects nucleotide sequences for siRNA synthesis based on the following protocol.

Selection of siRNA Target Sites

1. Beginning with the AUG start codon of the transcript, scan downstream for AA dinucleotide sequences. Record the occurrence of each AA and the 3' adjacent 19 nucleotides as potential siRNA target sites. Tuschl *et al.*, *Genes Dev* 13(24): 3191-7 (1999), don't recommend designing siRNA to the 5' and 3' untranslated regions (UTRs) and regions near the start codon (within 75bases) as these may be richer in regulatory protein binding sites. UTR-binding proteins and/or translation initiation complexes may interfere with binding of the siRNA endonuclease complex.
2. Compare the potential target sites to the appropriate genome database (human, mouse, rat, *etc.*) and eliminate from consideration any target sequences with significant homology to other coding sequences. It is suggested to use BLAST, which can be

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found on the NCBI server at: www.ncbi.nlm.nih.gov/BLAST/

3. Select qualifying target sequences for synthesis. Selecting several target sequences along the length of the gene to evaluate is typical.

Also included in the invention are isolated nucleic acid molecules that include the
5 nucleic acid sequence of target sequences, for example, nucleotide of SEQ ID NO: 5 or SEQ ID NO: 32, or a nucleic acid molecule that is complementary to the nucleic acid sequence of nucleotide of SEQ ID NO: 5 or SEQ ID NO: 32. As used herein, an "isolated nucleic acid" is a nucleic acid removed from its original environment (*e.g.*, the natural environment if naturally occurring) and thus, synthetically altered from its natural state. In the present
10 invention, isolated nucleic acid includes DNA, RNA, and derivatives thereof. When the isolated nucleic acid is RNA or derivatives thereof, base "t" should be replaced with "u" in the nucleotide sequences.

As used herein, the term "complementary" refers to Watson-Crick or Hoogsteen base pairing between nucleotides units of a nucleic acid molecule, and the term "binding"
15 means the physical or chemical interaction between two nucleic acids or compounds or associated nucleic acids or compounds or combinations thereof. When the polynucleotide comprises modified nucleotides and/or non-phosphodiester linkages, these polynucleotides may also bind each other as same manner. Generally, complementary nucleic acid sequences hybridize under appropriate conditions to form stable duplexes containing few or
20 no mismatches. For the purposes of this invention, two sequences having 5 or fewer mismatches are considered to be complementary. Furthermore, the sense strand and antisense strand of the isolated nucleotide of the present invention, can form double stranded nucleotide or hairpin loop structure by the hybridization.

In a preferred embodiment, such duplexes contain no more than 1 mismatch for
25 every 10 matches. In an especially preferred embodiment, where the strands of the duplex are fully complementary, such duplexes contain no mismatches. The nucleic acid molecule is less than 1518 nucleotides in length for REG4 or less than 1508 nucleotides in length for KIAA0101. For example, the nucleic acid molecule is less than about 500, about 200, or about 75 nucleotides in length. Also included in the invention is a vector containing one or
30 more of the nucleic acids described herein, and a cell containing the vectors. The isolated nucleic acids of the present invention are useful for siRNA against REG4 or KIAA0101, or DNA encoding the siRNA. When the nucleic acids are used for siRNA or coding DNA thereof, the sense strand is preferably longer than about 19 nucleotides, and more preferably

longer than 21 nucleotides.

The double-stranded molecules of the invention may contain one or more modified nucleotides and/or non-phosphodiester linkages. Chemical modifications well known in the art are capable of increasing stability, availability, and/or cell uptake of the double-stranded molecule. The skilled person will be aware of other types of chemical modification which may be incorporated into the present molecules (WO03/070744; WO2005/045037). In one embodiment, modifications can be used to provide improved resistance to degradation or improved uptake. Examples of such modifications include phosphorothioate linkages, 2'-O-methyl ribonucleotides (especially on the sense strand of a double-stranded molecule), 2'-deoxy-fluoro ribonucleotides, 2'-deoxy ribonucleotides, "universal base" nucleotides, 5'-C-methyl nucleotides, and inverted deoxyabasic residue incorporation (US20060122137).

In another embodiment, modifications can be used to enhance the stability or to increase targeting efficiency of the double-stranded molecule. Modifications include chemical cross linking between the two complementary strands of a double-stranded molecule, chemical modification of a 3' or 5' terminus of a strand of a double-stranded molecule, sugar modifications, nucleobase modifications and/or backbone modifications, 2-fluoro modified ribonucleotides and 2'-deoxy ribonucleotides (WO2004/029212). In another embodiment, modifications can be used to increased or decreased affinity for the complementary nucleotides in the target mRNA and/or in the complementary double-stranded molecule strand (WO2005/044976). For example, an unmodified pyrimidine nucleotide can be substituted for a 2-thio, 5-alkynyl, 5-methyl, or 5-propynyl pyrimidine. Additionally, an unmodified purine can be substituted with a 7-deza, 7-alkyl, or 7-alkenyl purine. In another embodiment, when the double-stranded molecule is a double-stranded molecule with a 3' overhang, the 3'-terminal nucleotide overhanging nucleotides may be replaced by deoxyribonucleotides (Elbashir SM *et al.*, Genes Dev 2001 Jan 15, 15(2): 188-200). For further details, published documents such as US20060234970 are available. The present invention is not limited to these examples and any known chemical modifications may be employed for the double-stranded molecules of the present invention so long as the resulting molecule retains the ability to inhibit the expression of the target gene.

Furthermore, the double-stranded molecules of the invention may comprise both DNA and RNA, *e.g.*, dsD/R-NA or shD/R-NA. Specifically, a hybrid polynucleotide of a DNA strand and an RNA strand or a DNA-RNA chimera polynucleotide shows increased stability. Mixing of DNA and RNA, *i.e.*, a hybrid type double-stranded molecule consisting

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of a DNA strand (polynucleotide) and an RNA strand (polynucleotide), a chimera type double-stranded molecule comprising both DNA and RNA on any or both of the single strands (polynucleotides), or the like may be formed for enhancing stability of the double-stranded molecule. The hybrid of a DNA strand and an RNA strand may be the hybrid in which either the sense strand is DNA and the antisense strand is RNA, or the opposite so long as it has an activity to inhibit expression of the target gene when introduced into a cell expressing the gene. Preferably, the sense strand polynucleotide is DNA and the antisense strand polynucleotide is RNA. Also, the chimera type double-stranded molecule may be either where both of the sense and antisense strands are composed of DNA and RNA, or where one of the sense and antisense strands is composed of DNA and RNA so long as it has an activity to inhibit expression of the target gene when introduced into a cell expressing the gene.

In order to enhance stability of the double-stranded molecule, the molecule preferably contains as much DNA as possible, whereas to induce inhibition of the target gene expression, the molecule is required to be RNA within a range to induce sufficient inhibition of the expression. As a preferred example of the chimera type double-stranded molecule, an upstream partial region (*i.e.*, a region flanking to the target sequence or complementary sequence thereof within the sense or antisense strands) of the double-stranded molecule is RNA. Preferably, the upstream partial region indicates the 5' side (5'-end) of the sense strand and the 3' side (3'-end) of the antisense strand. That is, in preferable embodiments, a region flanking to the 3'-end of the antisense strand, or both of a region flanking to the 5'-end of sense strand and a region flanking to the 3'-end of antisense strand consists of RNA. For instance, the chimera or hybrid type double-stranded molecule of the present invention comprise following combinations.

sense strand:	5'-[DNA]-3'
	3'-(RNA)-[DNA]-5' : antisense strand,
sense strand:	5'-(RNA)-[DNA]-3'
	3'-(RNA)-[DNA]-5' : antisense strand, and
sense strand:	5'-(RNA)-[DNA]-3'
	3'-(RNA)-5' : antisense strand.

The upstream partial region preferably is a domain consisting of 9 to 13 nucleotides counted from the terminus of the target sequence or complementary sequence thereto within the sense or antisense strands of the double-stranded molecules. Moreover, preferred

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examples of such chimera type double-stranded molecules include those having a strand length of 19 to 21 nucleotides in which at least the upstream half region (5' side region for the sense strand and 3' side region for the antisense strand) of the polynucleotide is RNA and the other half is DNA. In such a chimera type double-stranded molecule, the effect to inhibit expression of the target gene is much higher when the entire antisense strand is RNA (US20050004064).

In the present invention, the double-stranded molecule may form a hairpin, such as a short hairpin RNA (shRNA) and short hairpin consisting of DNA and RNA (shD/R-NA). The shRNA or shD/R-NA is a sequence of RNA or mixture of RNA and DNA making a tight hairpin turn that can be used to silence gene expression via RNA interference. The shRNA or shD/R-NA comprises the sense target sequence and the antisense target sequence on a single strand wherein the sequences are separated by a loop sequence. Generally, the hairpin structure is cleaved by the cellular machinery into dsRNA or dsD/R-NA, which is then bound to the RNA-induced silencing complex (RISC). This complex binds to and cleaves mRNAs which match the target sequence of the dsRNA or dsD/R-NA.

The invention is based in part on the discovery that the gene encoding REG4 is over-expressed in pancreatic ductal adenocarcinoma (PDACa) compared to non-cancerous pancreatic tissue, and that the gene encoding KIA0101 is over-expressed in pancreatic cancer, prostatic cancer cell, breast cancer cell, and bladder cancer cell compared to non-cancerous each tissue. The cDNA of REG4 is 1518 nucleotides in length. On the other hand, the cDNA of KIAA0101 is 1508 nucleotides in length. The nucleic acid and polypeptide sequences of REG4 (Genbank accession No: AY126670) are shown in SEQ ID NO: 1 and 2, respectively, and that of KIAA0101 (Genbank accession No: NM_014736) are shown in SEQ ID NO: 39 and 40, respectively.

The present invention discloses that transfection of siRNA comprising SEQ ID NO: 5 resulted in growth inhibition of PDAC cell lines. Furthermore, transfection of siRNA comprising SEQ ID NO: 32 resulted in a growth inhibition of pancreatic cancer cell lines.

Methods of inhibiting cell growth

The present invention relates to inhibiting cell growth, *i.e.*, cancer cell growth by inhibiting expression of REG4 or KIAA0101. Expression of REG4 or KIAA0101 is inhibited, for example, by small interfering RNA (siRNA) that specifically target the REG4 gene or KIAA0101 gene. REG4 targets include, for example, nucleotide of SEQ ID NO: 5, and KIAA0101 targets similarly include nucleotide of SEQ ID NO: 32.

The term “specifically inhibit” in the context of inhibitory polynucleotides and polypeptides refers to the ability of an agent or ligand to inhibit the expression or the biological function of REG4 and/or KIAA0101. Specific inhibition typically results in at least about a 2-fold inhibition over background, preferably greater than about 10-fold and most preferably greater than 100-fold inhibition of REG4 and/or KIAA0101 expression (*e.g.*, transcription or translation) or measured biological function (*e.g.*, cell growth or proliferation, inhibition of apoptosis, intracellular signaling from REG4, for example, activation of the EGF receptor/Akt/AP-1 signaling pathway with respect to REG4; binding to PCNA or another intracellular protein with respect to KIAA0101). Expression levels and/or biological function can be measured in the context of comparing treated and untreated cells, or a cell population before and after treatment. In some embodiments, the expression or biological function of REG4 and/or KIAA0101 is completely inhibited. Typically, specific inhibition is a statistically meaningful reduction in REG4/KIAA0101 expression or biological function (*e.g.*, $p \leq 0.05$) using an appropriate statistical test.

In non-mammalian cells, double-stranded RNA (dsRNA) has been shown to exert a strong and specific silencing effect on gene expression, which is referred as RNA interference (RNAi) (Sharp PA. *Genes Dev.* 1999 Jan 15;13(2):139-41.). dsRNA is processed into 20-23 nucleotides dsRNA called small interfering RNA (siRNA) by an enzyme containing RNase III motif. The siRNA specifically targets complementary mRNA with a multicomponent nuclease complex (Hammond SM, *et al.* *Nature.* 2000 Mar 16; 404 (6775): 293- 6; Hannon GJ. *Nature.* 2002 Jul 11; 418 (6894): 244-51.). In mammalian cells, siRNA composed of 20 or 21-mer dsRNA with 19 complementary nucleotides and 3' terminal noncomplementary dimers of thymidine or uridine, have been shown to have a gene specific knock-down effect without inducing global changes in gene expression (Elbashir SM, *et al.* *Nature.* 2001 May 24;411(6836):494-8.).

The growth of cells is inhibited by contacting a cell, with a composition containing a siRNA of REG4 or KIAA0101. The cell is further contacted with a transfection agent. Suitable transfection agents are known in the art. By inhibition of cell growth is meant the cell proliferates at a lower rate or has decreased viability compared to a cell not exposed to the composition. Cell growth is measured by methods known in the art such as, the MTT cell proliferation assay.

The siRNA of REG4 or KIAA0101 is directed to a single target of REG4 gene

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sequence or KIAA0101 gene sequence, respectively. Alternatively, the siRNA is directed to multiple targets of REG4 or KIAA0101 gene sequences. For example, the composition contains siRNA of REG4 or KIAA0101 directed to two, three, four, or five or more target sequences of REG4 or KIAA0101, respectively. By REG4 or KIAA0101 target sequence is meant a nucleotide sequence that is identical to a portion of the REG4 gene or the KIAA0101 gene (*i.e.*, a polynucleotide within a REG4 or KIAA0101 gene that is equal in length to and complementary to an siRNA). The target sequence can include the 5' untranslated (UT) region, the open reading frame (ORF) or the 3' untranslated region of the human REG4 or KIAA0101 gene. Alternatively, the siRNA is a nucleic acid sequence complementary to an upstream or downstream modulator of REG4 or KIAA0101 gene expression. Examples of upstream and downstream modulators include, a transcription factor that binds the REG4 or KIAA0101 gene promoter, a kinase or phosphatase that interacts with the REG4 or KIAA0101 polypeptide, a REG4 or KIAA0101 promoter or enhancer.

The siRNA of REG4 or KIAA0101 which hybridize to target mRNA decreases or inhibits production of the REG4 or KIAA0101 polypeptide product encoded by the REG4 or KIAA0101 gene by associating with the normally single-stranded mRNA transcript, thereby interfering with translation and thus, suppresses the expression of the protein. Thus, the siRNA molecules of the invention can be defined by their ability to hybridize specifically to mRNA or cDNA from a REG4 or KIAA0101 gene under stringent conditions.

For the purposes of this invention the terms "hybridize" or "hybridize specifically" are used to refer the ability of two nucleic acid molecules to hybridize under "stringent hybridization conditions." The phrase "stringent hybridization conditions" refers to conditions under which a nucleic acid molecule will hybridize to its target sequence, typically in a complex mixture of nucleic acids, but not detectably to other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m , 50% of the

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probes are occupied at equilibrium). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization. Exemplary stringent hybridization conditions can be as following: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with wash in 0.2x SSC, and 0.1% SDS at 50°C.

The siRNA of the invention is less than about 500, about 200, about 100, about 50, or about 25 nucleotides in length. Preferably the siRNA is about 19 to about 25 nucleotides in length. Exemplary nucleic acid sequence for the production of REG4 siRNA includes the sequence of nucleotide of SEQ ID NO: 5 as the target sequence. Similarly, nucleic acid sequence for the production of KIAA0101 siRNA include the sequence of nucleotide of SEQ ID NO:32 as the target sequence. Furthermore, in order to enhance the inhibition activity of the siRNA, nucleotide "u" can be added to 3' end of the antisense strand of the target sequence. The number of "u"s to be added is at least about 2, generally about 2 to about 10, preferably about 2 to about 5. The added "u"s form single strand at the 3' end of the antisense strand of the siRNA.

The cell is any cell that expresses or over-expresses REG4 or KIAA0101. The cell is an epithelial cell such as a pancreatic ductal cell. Alternatively, the cell is a tumor cell such as a carcinoma, adenocarcinoma, blastoma, leukemia, myeloma, or sarcoma. The cell is a pancreatic cancer cell, especially pancreatic ductal adenocarcinoma cell, prostatic cancer cell, breast cancer cell, and bladder cancer cell.

The siRNA of REG4 or KIAA0101 is directly introduced into the cells in a form that is capable of binding to the mRNA transcripts. Alternatively, the DNA encoding the siRNA of REG4 or KIAA0101 is in a vector.

Vectors are produced for example by cloning a REG4 or KIAA0101 target sequence into an expression vector operatively-linked regulatory sequences flanking the REG4 or KIAA0101 sequence in a manner that allows for expression (by transcription of the DNA molecule) of both strands (Lee, N.S., *et al.* Nature Biotechnology 20 : 500-5.). An RNA molecule that is antisense to REG4 or KIAA0101 mRNA is transcribed by a first promoter (*e.g.*, a promoter sequence 3' of the cloned DNA) and an RNA molecule that is the sense strand for the REG4 or KIAA0101 mRNA is transcribed by a second promoter (*e.g.*, a promoter sequence 5' of the cloned DNA). The sense and antisense strands hybridize *in vivo* to generate the siRNA constructs for silencing of the REG4 or KIAA0101 gene.

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Alternatively, two constructs are utilized to create the sense and anti-sense strands of a siRNA construct. Cloned REG4 or KIAA0101 can encode a construct having secondary structure, *e.g.*, hairpins, wherein a single transcript has both the sense and complementary antisense sequences from the target gene.

5 A loop sequence consisting of an arbitrary nucleotide sequence can be located between the sense and antisense sequence in order to form the hairpin loop structure. Thus, the present invention also provides the siRNA having the general formula 5'-[A]-[B]-[A']-3', wherein [A] is a ribonucleotide sequence corresponding to a sequence that specifically hybridizes to an mRNA or a cDNA from REG4 or KIAA0101. In preferred embodiments,
10 [A] is a ribonucleotide sequence corresponding to a sequence of nucleotides of SEQ ID NO: 5 or SEQ ID NO:32.

[B] is a ribonucleotide sequence consisting of 3 to 23 nucleotides, and

[A'] is a ribonucleotide sequence consisting of the complementary sequence of [A]

The region [A] hybridizes to [A'], and then a loop consisting of region [B] is formed.

15 The loop sequence may be preferably about 3 to about 23 nucleotide in length. The loop sequence, for example, can be selected from group consisting of following sequences (www.ambion.com/techlib/tb/tb_506.html). Furthermore, loop sequence consisting of 23 nucleotides also provides active siRNA (Jacque, J.M., *et al.* (2002) Nature 418 : 435-438.).

CCC, CCACC or CCACACC: Jacque, J.M., *et al.* (2002) Nature, Vol. 418: 435-8.

20 UUCG: Lee, N.S., *et al.* (2002) Nature Biotechnology 20 : 500-5. Fruscoloni, P., *et al.* (2003) Proc. Natl. Acad. Sci. USA 100(4): 1639-44.

UUCAAGAGA: Dykxhoorn, D. M., *et al.* Nature Reviews Molecular Cell Biology 4: 457-67.

For example, preferable siRNAs having hairpin loop structure of the present invention
25 are shown below. In the following structure, the loop sequence can be selected from group consisting of CCC, UUCG, CCACC, CCACACC, and UUCAAGAGA. Preferable loop sequence is UUCAAGAGA ("ttcaagaga" in DNA).

5'-GACAGAAGGAAGAACTCA-[B]- TGAGTTTCTTCCTTCTGTC-3' (for target
sequence of SEQ ID NO:5)

30 The regulatory sequences flanking the REG4 or KIAA0101 sequence are identical or are different, such that their expression can be modulated independently, or in a temporal or spatial manner. siRNAs are transcribed intracellularly by cloning the REG4 or KIAA0101

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gene templates into a vector containing, *e.g.*, a RNA polymerase III transcription unit from the small nuclear RNA (snRNA) U6 or the human H1 RNA promoter. For introducing the vector into the cell, transfection-enhancing agent can be used. FuGENE (Roche Diagnostics), Lipofectamine 2000 (Invitrogen), Oligofectamine (Invitrogen), and

5 Nucleofector (Wako pure Chemical) are useful as the transfection-enhancing agent.

Oligonucleotides and oligonucleotides complementary to various portions of REG4 or KIAA0101 mRNA were tested *in vitro* for their ability to decrease production of REG4 or KIAA0101 in tumor cells (*e.g.*, using the pancreatic cell line such as pancreatic cancer cell line or pancreatic ductal adenocarcinoma (PDAC) cell line) according to standard methods.

10 A reduction in REG4 or KIAA0101 gene product in cells contacted with the candidate siRNA composition compared to cells cultured in the absence of the candidate composition is detected using specific antibodies of REG4 or KIAA0101, or other detection strategies. Sequences which decrease production of REG4 or KIAA0101 in *in vitro* cell-based or cell-free assays are then tested for their inhibitory effects on cell growth. Sequences which

15 inhibit cell growth *in vitro* cell-based assay are test *in vivo* in rats or mice to confirm decreased REG4 or KIAA0101 production and decreased tumor cell growth in animals with malignant neoplasms.

Methods of treating malignant tumors

Patients with tumors characterized as over-expressing REG4 or KIAA0101 are

20 treated by administering siRNA of REG4 or KIAA0101, respectively. siRNA therapy is used to inhibit expression of REG4 or KIAA0101 in patients suffering from or at risk of developing, for example, pancreatic cancer, especially pancreatic ductal adenocarcinoma (PDAC), prostatic cancer cell, breast cancer cell, and bladder cancer cell. Such patients are identified by standard methods of the particular tumor type. Pancreatic cancer, pancreatic

25 ductal adenocarcinoma (PDAC), prostatic cancer cell, breast cancer cell, and bladder cancer cell is diagnosed for example, by CT, MRI, ERCP, MRCP, computer tomography, or ultrasound. Treatment is efficacious if the treatment leads to clinical benefit such as, a reduction in expression of REG4 or KIAA0101, or a decrease in size, prevalence, or metastatic potential of the tumor in the subject. When treatment is applied prophylactically,

30 "efficacious" means that the treatment retards or prevents tumors from forming or prevents or alleviates a clinical symptom of the tumor. Efficaciousness is determined in association with any known method for diagnosing or treating the particular tumor type.

siRNA therapy is carried out by administering to a patient an siRNA by standard

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vectors encoding the siRNAs of the invention and/or gene delivery systems such as by delivering the synthetic siRNA molecules. Typically, synthetic siRNA molecules are chemically stabilized to prevent nuclease degradation *in vivo*. Methods for preparing chemically stabilized RNA molecules are well known in the art. Typically, such molecules
5 comprise modified backbones and nucleotides to prevent the action of ribonucleases. Other modifications are also possible, for example, cholesterol-conjugated siRNAs have shown improved pharmacological properties. (Song *et al. Nature Med.* 9:347-351 (2003)).

Suitable gene delivery systems may include liposomes, receptor-mediated delivery systems, or viral vectors such as herpes viruses, retroviruses, adenoviruses and adeno-
10 associated viruses, among others. A therapeutic nucleic acid composition is formulated in a pharmaceutically acceptable carrier. The therapeutic composition may also include a gene delivery system as described above. Pharmaceutically acceptable carriers are biologically compatible vehicles which are suitable for administration to an animal, *e.g.*, physiological saline. A therapeutically effective amount of a compound is an amount which is capable of
15 producing a medically desirable result such as reduced production of a REG4 or KIAA0101 gene product, reduction of cell growth, *e.g.*, proliferation, or a reduction in tumor growth in a treated animal.

Parenteral administration, such as intravenous, subcutaneous, intramuscular, and intraperitoneal delivery routes, may be used to deliver siRNA compositions of REG4 or
20 KIAA0101. For treatment of pancreatic, prostatic, breast, and bladder tumors, direct infusion into the tissue or near the site of cancer, is useful.

Dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular nucleic acid to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Dosage for
25 intravenous administration of nucleic acids is from approximately 10^6 to 10^{22} copies of the nucleic acid molecule.

The polynucleotides are administered by standard methods, such as by injection into the interstitial space of tissues such as muscles or skin, introduction into the circulation or into body cavities or by inhalation or insufflation. Polynucleotides are injected or otherwise
30 delivered to the animal with a pharmaceutically acceptable liquid carrier, *e.g.*, a liquid carrier, which is aqueous or partly aqueous. The polynucleotides are associated with a liposome (*e.g.*, a cationic or anionic liposome). The polynucleotide includes genetic information necessary for expression by a target cell, such as a promoter.

Anti-REG4 Antibodies and Antigen Binding Proteins:**Antibodies**

The present inventors have shown that a monoclonal antibody against REG4 neutralized its growth-promoting effects of REG4 and attenuated significantly the growth of PDAC cells. The results revealed that treatment of disease associated with REG4-expressing cells, for example, pancreatic cancer is conveniently carried out using antibodies that bind to REG4.

The present invention relates to pharmaceutical compositions for treating or preventing cancers mediated by aberrant over-expression of REG4, including pancreatic cancer, said composition comprising a pharmaceutically effective amount of an antibody or fragment thereof, or an antigen binding protein, that binds to a protein encoded by REG4 as an active ingredient, and a pharmaceutically acceptable carrier. The present invention also relates to use of an anti-REG4 antibody or antigen binding protein to produce pharmaceutical compositions for treating or preventing pancreatic cancer. The pharmaceutical compositions of the present invention comprise anti-REG4 antibodies or antigen binding proteins and pharmaceutically acceptable carriers.

An "isolated" or "purified" polypeptide is a polypeptide that is substantially free of cellular material such as carbohydrate, lipid, or other contaminating proteins from the cell or tissue source from which the protein is derived, or substantially free of chemical precursors or other chemicals when chemically synthesized. The term "substantially free of cellular material" includes preparations of a polypeptide in which the polypeptide is separated from cellular components of the cells from which it is isolated or recombinantly produced.

Thus, a polypeptide that is substantially free of cellular material includes preparations of polypeptide having less than about 30%, 20%, 10%, or 5% (by dry weight) of heterologous protein (also referred to herein as a "contaminating protein"). When the polypeptide is recombinantly produced, it is also preferably substantially free of culture medium, which includes preparations of polypeptide with culture medium less than about 20%, 10%, or 5% of the volume of the protein preparation. When the polypeptide is produced by chemical synthesis, it is preferably substantially free of chemical precursors or other chemicals, which includes preparations of polypeptide with chemical precursors or other chemicals involved in the synthesis of the protein less than about 30%, 20%, 10%, 5% (by dry weight) of the volume of the protein preparation. That a particular protein preparation

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contains an isolated or purified polypeptide can be shown, for example, by the appearance of a single band following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the protein preparation and Coomassie Brilliant Blue staining of the gel. In a preferred embodiment, antibodies of the present invention or fragments thereof are isolated or purified.

5 An "isolated" or "purified" nucleic acid molecule is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid molecule. An "isolated" or "purified" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when
10 chemically synthesized. In a preferred embodiment, nucleic acid molecules encoding antibodies of the present invention or fragments thereof are isolated or purified.

"Antibodies" and "immunoglobulins" are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules, for which
15 antigen specificity has not been defined. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies and immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent
20 disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (VH) followed by a number of constant domains (CH). Each light chain has a variable domain at one end (VL) and a constant domain at its other end (CL); the constant domain of the light
25 chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains (Chothia *et al.*, (1985) J Mol Biol.;186:651-63; Novotny and Haber, (1985) Proc Natl Acad Sci USA.;82:4592-6).

30 The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly

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distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four framework regions, largely adopting a β -sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of the β -sheet structure. The CDRs in each chain are held together in close proximity by the framework regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (Kabat *et al.*, (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, National Institute of Health, Bethesda, Md.). The constant domains are not involved directly in binding an antibody to an antigen but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-binding sites. "Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH-1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH-1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab', in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

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The “light chains” of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called κ (kappa) and λ (lambda), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these can be further divided into subclasses (isotypes), *e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α , δ , ϵ , γ , and μ , respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term “monoclonal antibody” as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they can be synthesized by hybridoma culture, uncontaminated by other immunoglobulins. Thus, the modifier “monoclonal” indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention can be made by the hybridoma method first described by Kohler and Milstein, (1975) *Nature*, 256:495-7, or can be made by recombinant DNA methods (Cabilly *et al.*, (1984) *Proc Natl Acad Sci USA*, 81:3273-7).

The monoclonal antibodies herein specifically include “chimeric” antibodies or immunoglobulins, in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (Cabilly *et al.*, *supra*;

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Morrison *et al.*, (1984) *Proc Natl Acad Sci USA*.;81:6851-5). Most typically, chimeric antibodies or immunoglobulins comprise human and murine antibody fragments, generally human constant and mouse variable regions.

“Humanized” forms of non-human (*e.g.*, murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains or fragments thereof which contain minimal sequence derived from non-human immunoglobulin. Such fragments also includes Fv, Fab, Fab’, F(ab’)₂, or other antigen-binding subsequences of antibodies. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity-determining region (CDR) of the recipient are replaced by residues derived from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity.

In some instances, Fv framework residues of the human immunoglobulin may be replaced by corresponding non-human residues. In the present invention, few, two, or preferably one of framework(s) in the humanized antibody may be replaced by that of non-human residues. Furthermore, humanized antibodies can comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the framework regions are those of a human immunoglobulin consensus sequence.

The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details *see* Jones *et al.*, (1986) *Nature*.;321:522-5; Riechmann *et al.*, (1988) *Nature*.;332:323-7; Presta, (1992) *Curr Opin Struct Biol*. 2:593-6.

“Single-chain Fv” or “sFv” antibody fragments comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the sFv to form the desired structure for antigen binding. A number of methods have been described to discern chemical structures for converting the naturally aggregated but chemically separated light and heavy polypeptide chains from an antibody V region into an sFv molecule which will fold into a three dimensional structure substantially

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similar to the structure of an antigen-binding site (U.S. Pat. Nos. 5,091,513, 5,132,405, and 4,946,778; Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994)).

Antigen Binding Proteins

5 The present invention also includes antigen binding proteins or non-antibody binding proteins (*e.g.*, ligands) that specifically bind to REG4 or KIAA0101. Non-antibody ligands include antibody mimics that use non-immunoglobulin protein scaffolds, including adnectins, avimers, single chain polypeptide binding molecules, and antibody-like binding peptidomimetics, as discussed in more detail below.

10 Other compounds have been developed that target and bind to targets in a manner similar to antibodies. Certain of these "antibody mimics" use non-immunoglobulin protein scaffolds as alternative protein frameworks for the variable regions of antibodies.

 For example, Ladner *et al.* (U.S. Patent No. 5,260,203) describe single polypeptide chain binding molecules with binding specificity similar to that of the aggregated, but
15 molecularly separate, light and heavy chain variable region of antibodies. The single-chain binding molecule contains the antigen binding sites of both the heavy and light variable regions of an antibody connected by a peptide linker and will fold into a structure similar to that of the two peptide antibody. The single-chain binding molecule displays several advantages over conventional antibodies, including, smaller size, greater stability and are
20 more easily modified.

 Ku *et al.* (*Proc. Natl. Acad. Sci. U.S.A.* 92(14):6552-6556 (1995)) discloses an alternative to antibodies based on cytochrome b562. Ku *et al.* (1995) generated a library in which two of the loops of cytochrome b562 were randomized and selected for binding against bovine serum albumin. The individual mutants were found to bind selectively with BSA
25 similarly with anti-BSA antibodies.

 Lipovsek *et al.* (U.S. Patent Nos. 6,818,418 and 7,115,396) discloses an antibody mimic featuring a fibronectin or fibronectin-like protein scaffold and at least one variable loop. Known as Adnectins, these fibronectin-based antibody mimics exhibit many of the same characteristics of natural or engineered antibodies, including high affinity and specificity for
30 any targeted ligand. Any technique for evolving new or improved binding proteins can be used with these antibody mimics.

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The structure of these fibronectin-based antibody mimics is similar to the structure of the variable region of the IgG heavy chain. Therefore, these mimics display antigen binding properties similar in nature and affinity to those of native antibodies. Further, these fibronectin-based antibody mimics exhibit certain benefits over antibodies and antibody fragments. For example, these antibody mimics do not rely on disulfide bonds for native fold stability, and are, therefore, stable under conditions which would normally break down antibodies. In addition, since the structure of these fibronectin-based antibody mimics is similar to that of the IgG heavy chain, the process for loop randomization and shuffling can be employed in vitro that is similar to the process of affinity maturation of antibodies in vivo.

Beste *et al.* (Proc. Natl. Acad. Sci. U.S.A. 96(5):1898-1903 (1999)) discloses an antibody mimic based on a lipocalin scaffold (Anticalin®). Lipocalins are composed of a β -barrel with four hypervariable loops at the terminus of the protein. Beste (1999), subjected the loops to random mutagenesis and selected for binding with, for example, fluorescein. Three variants exhibited specific binding with fluorescein, with one variant showing binding similar to that of an anti-fluorescein antibody. Further analysis revealed that all of the randomized positions are variable, indicating that Anticalin® would be suitable to be used as an alternative to antibodies.

Anticalins® are small, single chain peptides, typically between 160 and 180 residues, which provides several advantages over antibodies, including decreased cost of production, increased stability in storage and decreased immunological reaction.

Hamilton *et al.* (U.S. Patent No. 5,770,380) discloses a synthetic antibody mimic using the rigid, non-peptide organic scaffold of calixarene, attached with multiple variable peptide loops used as binding sites. The peptide loops all project from the same side geometrically from the calixarene, with respect to each other. Because of this geometric confirmation, all of the loops are available for binding, increasing the binding affinity to a ligand. However, in comparison to other antibody mimics, the calixarene-based antibody mimic does not consist exclusively of a peptide, and therefore it is less vulnerable to attack by protease enzymes. Neither does the scaffold consist purely of a peptide, DNA or RNA, meaning this antibody mimic is relatively stable in extreme environmental conditions and has a long life span. Further, since the calixarene-based antibody mimic is relatively small, it is less likely to produce an immunogenic response.

Murali *et al.* (Cell. Mol. Biol. 49(2):209-216 (2003)) discusses a methodology for

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reducing antibodies into smaller peptidomimetics, they term "antibody like binding peptidomemetics" (ABiP) which can also be useful as an alternative to antibodies.

Silverman *et al.* (*Nat. Biotechnol.* (2005), 23: 1556-1561) discloses fusion proteins that are single-chain polypeptides comprising multiple domains termed "avimers."

- 5 Developed from human extracellular receptor domains by *in vitro* exon shuffling and phage display the avimers are a class of binding proteins somewhat similar to antibodies in their affinities and specificities for various target molecules. The resulting multidomain proteins can comprise multiple independent binding domains that can exhibit improved affinity (in some cases sub-nanomolar) and specificity compared with single-epitope binding proteins.
- 10 Additional details concerning methods of construction and use of avimers are disclosed, for example, in U.S. Patent App. Pub. Nos. 20040175756, 20050048512, 20050053973, 20050089932 and 20050221384.

- In addition to non-immunoglobulin protein frameworks, antibody properties have also been mimicked in compounds comprising RNA molecules and unnatural oligomers (e.g.,
- 15 protease inhibitors, benzodiazepines, purine derivatives and beta-turn mimics) all of which are suitable for use with the present invention.

Neutralizing activity

- Antibodies and non-antibody binding proteins exist which have the function of depriving infectivity of pathogens and activity of toxins. Antibody-mediated neutralization
- 20 can be achieved by binding of an antigenic variable region to an antigen, or can require complement mediation. For example, in some cases, anti-viral antibodies require complement mediation in order to deprive a virus of its infectivity. Fc regions are essential to the participation of complements. Thus, such antibodies comprise effector function that requires Fc for neutralizing viruses and cells.

- 25 The present invention is based in part on the finding that anti-REG4 antibodies and non-antibody binding proteins specifically bind to REG4, and then neutralize REG4 activity promoting cell proliferation, particularly in cancer cells where proliferation is mediated by abnormally high REG4 expression or intracellular signalling.

- The terms "bind(s) specifically" or "specifically bind(s)" or "attached" or "attaching"
- 30 in the context of antibodies or non-antibody binding proteins refers to the preferential association of an agent or ligand, in whole or part, with a target epitope (e.g. REG4) that binds or competes with another agent or ligand for binding to REG4 expressed on a cell or tissue.

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It is, of course, recognized that a certain degree of non-specific interaction may occur between an antibody and a non-target epitope. Nevertheless, specific binding, can be distinguished as mediated through specific recognition of the target epitope. Typically specific binding results in a much stronger association between the delivered molecule and an entity (*e.g.*, an assay well or a cell) bearing the target epitope than between the bound antibody and an entity (*e.g.*, an assay well or a cell) lacking the target epitope. Specific binding typically results in at least about a 2-fold increase over background, preferably greater than about 10-fold and most preferably greater than 100-fold increase in amount of bound agent or ligand (per unit time) to a cell or tissue bearing the target epitope (*i.e.* REG4) as compared to a cell or tissue lacking the target epitope. Specific binding between two entities generally means an affinity of at least 10^6 M^{-1} . Affinities greater than 10^8 M^{-1} or greater are preferred. Specific binding can be determined for nucleic acid as well as protein agents and ligands. Specific binding for nucleic acid agents can be determined using any assay known in the art, including but not limited to northern blots, gel shift assays and *in situ* hybridization. Specific binding for protein agents and ligands can be determined using any binding assay known in the art, including but not limited to gel electrophoresis, western blot, ELISA, flow cytometry, and immunohistochemistry.

The present invention also relates to methods for suppressing cell growth of REG4-expressing cells, which comprise the following steps:

- 1) contacting the REG4-expressing cells with anti-REG4 antibodies or anti-REG4 non-antibody binding proteins, and
- 2) neutralizing the cell proliferation activity of REG4.

In the methods or pharmaceutical compositions of the present invention, any REG4-expressing cell can be suppressed. For example, pancreatic cancer cells are preferable as the REG4-expressing cells of the present invention. Of these, pancreatic carcinoma or cells are preferable.

Cells and antibodies (or non-antibody binding proteins) can be contacted *in vivo* or *in vitro*. When targeting *in vivo* cancer cells as the REG4-expressing cells, the methods of the present invention are in fact therapeutic methods or preventative methods for cancers. Specifically, the present invention provides therapeutic methods for cancers which comprise the following steps:

- 1) administering an antibody or non-antibody binding protein that specifically

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binds REG4 to a cancer patient, and

- 2) suppressing cancer cell growth using the function of the antibody or non-antibody binding protein bound to REG4, wherein the function is neutralizing the cell proliferation activity of REG4.

5 In the present invention, neutralizing function of the anti-REG4 antibody or non-antibody binding protein refers to inhibition of REG4 activity for stimulating cell proliferation of REG4 expression cells. For instance, the present inventors confirm that REG4 functions as an autocrine or paracrine growth factor and mediate Akt signaling pathways. Accordingly, in the preferable embodiments of the present invention, anti-REG4 antibodies or non-antibody
10 binding proteins shut down the REG4 autocrine/paracrine pathway and block the subsequent Akt phosphorylation. The present inventors also confirmed that antibodies binding REG4 effectively suppress the cell proliferation of REG4-expressing cells, in particular, pancreatic cancer cells using neutralizing function. The present inventors further confirmed that REG4 is highly expressed in pancreatic cancer cells, with a high probability. In addition, REG4
15 expression levels in normal tissues are low. Putting this information together, methods of pancreatic cancer therapy where anti-REG4 antibody is administered can be effective, with little danger of side effects.

However, the antibodies and non-antibody binding proteins of the present invention are not limited so long as they comprise a desired neutralizing function. Variants, analogs or
20 derivatives of the Fc portion may be constructed by, for example, making various substitutions of residues or sequences.

Variant (or analog) polypeptides include insertion variants, wherein one or more amino acid residues supplement an Fc amino acid sequence. Insertions may be located at either or both termini of the protein, or may be positioned within internal regions of the Fc
25 amino acid sequence. Insertional variants with additional residues at either or both termini can include, for example, fusion proteins and proteins including amino acid tags or labels. For example, the Fc molecule may optionally contain an N-terminal Met, especially when the molecule is expressed recombinantly in a bacterial cell such as *E. coli*.

In Fc deletion variants, one or more amino acid residues in an Fc polypeptide are
30 removed. Deletions can be effected at one or both termini of the Fc polypeptide, or with removal of one or more residues within the Fc amino acid sequence. Deletion variants, therefore, include all fragments of an Fc polypeptide sequence.

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In Fc substitution variants, one or more amino acid residues of an Fc polypeptide are removed and replaced with alternative residues. In one aspect, the substitutions are conservative in nature, however, the invention embraces substitutions that are non-conservative.

5 Preferably, the parent polypeptide Fc region is a human Fc region, *e.g.*, a native sequence human Fc region human IgG1 (A and non-A allotypes) or human IgG3 Fc region. In one embodiment, the variant with improved ADCC mediates ADCC substantially more effectively than an antibody with a native sequence IgG1 or IgG3 Fc region and the antigen-binding region of the variant. Preferably, the variant comprises, or consists essentially of,
10 substitutions of two or three of the residues at positions 298, 333 and 334 of the Fc region. The numbering of the residues in an immunoglobulin heavy chain is that of the EU index as in Kabat *et al.*, (*supra*), expressly incorporated herein by reference. Most preferably, residues at positions 298, 333 and 334 are substituted, (*e.g.*, with alanine residues). Moreover, in order to generate the Fc region variant with improved ADCC activity, one will generally
15 engineer an Fc region variant with improved binding affinity for FcγRIII, which is thought to be an important FcR for mediating ADCC. For example, one may introduce an amino acid modification (*e.g.*, an insertion, a deletion, or a substitution) into the parent Fc region at any one or more of amino acid positions 256, 290, 298, 312, 326, 330, 333, 334, 360, 378 or 430 to generate such a variant. The variant with improved binding affinity for FcγRIII may
20 further have reduced binding affinity for FcγRII, especially reduced affinity for the inhibiting FcγRIIB receptor.

In any event, any variant amino acid insertions, deletions and/or substitutions (*e.g.*, from 1-50 amino acids, preferably, from 1-25 amino acids, more preferably, from 1-10 amino acids) are contemplated and are within the scope of the present invention. Conservative
25 amino acid substitutions will generally be preferred. Furthermore, alterations may be in the form of altered amino acids, such as peptidomimetics or D-amino acids.

Therefore, human-derived antibodies belonging to these classes are preferable in the present invention. Human antibodies can be acquired using antibody-producing cells harvested from humans, or chimeric animals transplanted with human antibody genes (Ishida
30 I, *et al.*, (2002) Cloning and Stem Cells., 4: 91-102.).

Furthermore, antibody Fc regions can link with arbitrary variable regions. Specifically, chimeric antibodies wherein the variable regions of different animal species are

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bound to human constant regions are known. Alternatively, a human-human chimeric antibody can also be acquired by binding human-derived variable regions to arbitrary constant regions. In addition, CDR graft technology, where complementarity determining regions (CDRs) composing human antibody variable regions are replaced with CDRs of heterologous antibodies, is also known ("Immunoglobulin genes", Academic Press (London), pp260-274, 1989; Roguska MA, *et al.*, (1994) Proc. Natl. Acad. Sci. USA., 91: 969-73.).

By replacing CDRs, antibody binding specificity is replaced. That is, human REG4 will be recognized by humanized antibodies in which the CDR of human REG4-binding antibodies has been transferred. The transferred antibodies can also be called humanized antibodies. Antibodies thus-obtained and equipped with an Fc region essential to effector function can be used as the antibodies of the present invention, regardless of the origin of their variable regions. For example, antibodies comprising a human IgG Fc are preferable in the present invention, even if their variable regions comprise an amino acid sequence derived from an immunoglobulin of another class or another species.

Alternatively, antibody fragment that lacks Fc region may be used so long as they comprise a desired neutralizing function. For example, in the present invention, Fv, Fab, Fab', F(ab')₂, or other antigen-binding subsequences of antibodies may also be used as antibody. In some embodiments, an agent to enhance the neutralizing effect may be conjugated with antibody, or fragment thereof.

VH and VL domains of antibodies of the present invention each comprise three CDRs designated as CDR1, CDR2, and CDR3 separated by framework regions. Amino acid sequences of the CDRs are not particularly limited as long as the antibody can specifically bind to REG4. Preferred examples of CDR amino acid sequences include:

VH CDR1 : SYWMH (SEQ ID NO: 20),

VH CDR2 : NIYPGSGSTNYD (SEQ ID NO: 21),

VH CDR3 : GGLWLRVDY (SEQ ID NO: 22),

VL CDR1 : SASSSVSYM (SEQ ID NO: 23),

VL CDR2 : DTSKLAS (SEQ ID NO: 24),

VL CDR3 : QQWSSNPF (SEQ ID NO: 25)

In a more preferred embodiment, VH comprises the amino acid sequence of SEQ ID NO: 18, and VL comprises the amino acid sequence of SEQ ID NO: 19. In some embodiments, the VH comprises an amino acid sequence having at least about 90%, 95%,

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98% sequence identity to the full-length of SEQ ID NO: 18, and the VL comprises an amino acid sequence having at least about 90%, 95%, 98% sequence identity to the full-length of SEQ ID NO: 19. Sequence identity can be measured using default settings of available software well known in the art, for example, BLAST or ALIGN.

5 In the present invention, the antibodies may be monoclonal antibodies or polyclonal antibodies. Even when administering to humans, human polyclonal antibodies can be derived using the above-mentioned animals transferred with a human antibody gene. Alternatively, immunoglobulins which have been constructed using genetic engineering techniques, such as humanized antibodies, human-non-human chimeric antibodies, and
10 human-human chimeric antibodies, can be used. Furthermore, methods for obtaining human monoclonal antibodies by cloning human antibody-producing cells are also known.

REG4, or a fragment comprising its partial peptide, can be used as immunogens to obtain the antibodies. In the present invention, REG4 can be derived from any species, preferably from a mammal such as a human, mouse, or rat, and more preferably from a human.

15 The human REG4 nucleotide sequence and amino acid sequence are known. The cDNA nucleotide sequence of REG4 is described in SEQ ID NO: 1 and the amino acid sequences coded by that nucleotide sequence is described in SEQ ID NO: 2 (GenBank Accession No. AY126670). One skilled in the art can routinely isolate genes comprising the provided nucleotide sequence, preparing a fragment of the sequence as required, and obtain a protein
20 comprising the target amino acid sequence.

For example, the gene coding the REG4 protein or its fragment can be inserted into a known expression vector, and used to transform host cells. The desired protein, or its fragment, can be collected from inside or outside host cells using arbitrary and standard methods, and can also be used as an antigen. In addition, proteins, their lysates, and
25 chemically-synthesized proteins can be used as antigens. Furthermore, cells expressing the REG4 protein or a fragment thereof can themselves be used as immunogens.

When using a peptide fragment as the REG4 immunogen, it is particularly preferable to select an amino acid sequence which comprises a region predicted to be an extra-cellular domain. The existence of a signal peptide is predicted from positions 1 to 25
30 on the N-terminal of REG4. Thus, for example, a region other than the N-terminal signal peptide (25 amino acid residues) is preferred as the immunogen for obtaining the antibodies of the present invention. That is to say, antibodies that bind to REG4 extra-cellular domains

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are preferred as the antibodies of the present invention.

Therefore, preferable antibodies in the present invention are antibodies equipped with an Fc essential to effector function, and a variable region that can bind to an extracellular REG4 domain. When aiming for administration to humans, it is preferable to be equipped with an IgG Fc.

Any mammal can be immunized with such an antigen. However, it is preferable to consider compatibility with parent cells used in cell fusion. Generally, rodents, lagomorphs, or primates are used.

Rodents include, for example, mice, rats, and hamsters. Lagomorphs include, for example, rabbits. Primates include, for example, catarrhine (old world) monkeys such as *Macaca fascicularis*, *Macaca mulatta*, Sacred baboons, and chimpanzees.

Methods for immunizing animals with antigens are well known in the field. Intraperitoneal or subcutaneous antigen injections are standard methods for immunizing mammals. Specifically, antigens can be diluted and suspended in an appropriate amount of phosphate buffered saline (PBS), physiological saline, or so on. As desired, antigen suspensions can be mixed with an appropriate amount of a standard adjuvant such as Freund's complete adjuvant, and administered to mammals after emulsification. Subsequently, it is preferable that antigens mixed with an appropriate amount of Freund's incomplete adjuvant are administered in multiple doses every four to 21 days. An appropriate carrier can also be used for immunization. After carrying out immunization as outlined above, standard methods can be used to examine serum for an increase in the desired antibody level.

Polyclonal antibodies against the REG4 protein can be prepared from immunized mammals whose serum has been investigated for an increase in the desired antibodies. This can be achieved by collecting blood from these animals, or by using an arbitrary, usual method to isolate serum from their blood. Polyclonal antibodies comprise serum that comprises polyclonal antibodies, and fractions that comprise polyclonal antibodies which can be isolated from serum. IgG and IgM can be prepared from fractions that recognize REG4 protein by using, for example, an affinity column coupled to REG4 protein, and then further purifying this fraction using protein A or protein G columns. In the present invention, antiserum can be used as is as polyclonal antibodies. Alternatively, purified IgG, IgM, or such can also be used.

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To prepare monoclonal antibodies, immunocytes are collected from mammals immunized with antigens, investigated for the increase of the desired antibody level in serum (as above), and applied in cell fusion. Immunocytes for use in cell fusion preferably come from the spleen. Other preferred parent cells for fusion with the above immunogens include, for example, mammalian myeloma cells, and more preferably, myeloma cells that have acquired properties for selection of fusion cells by pharmaceutical agents.

The above immunocytes and myeloma cells can be fused using known methods, for example the methods of Milstein *et al.* (Galfre, G. and Milstein, C., (1981) *Methods. Enzymol.* : 73, 3-46.).

Hybridomas produced by cell fusion can be selected by culturing in a standard selective medium such as HAT medium (medium comprising hypoxanthine, aminopterin, and thymidine). Cell culture in HAT medium is usually continued for several days to several weeks, a period sufficient enough to kill all cells other than the desired hybridomas (unfused cells). Standard limiting dilutions are then carried out, and hybridoma cells that produce the desired antibodies are screened and cloned.

Non-human animals can be immunized with antigens for preparing hybridomas in the above method. In addition, human lymphocytes from cells infected with EB virus or such, can be immunized *in vitro* using proteins, cells expressing proteins, or suspensions of the same. The immunized lymphocytes are then fused with human-derived myeloma cells able to divide unlimitedly (U266 and so on), thus obtaining hybridomas that produce the desired human antibodies which can bind the protein (Unexamined Published Japanese Patent Application No. (JP-A) Sho 63-17688).

The obtained hybridomas are then transplanted to mice abdominal cavities, and ascites are extracted. The obtained monoclonal antibodies can be purified using, for example, ammonium sulfate precipitation, protein A or protein G columns, DEAE ion exchange chromatography, or affinity columns coupled to the proteins of the present invention. The antibodies of the present invention can be used not only in purifying and detecting the proteins of the present invention, but also as candidates for agonists and antagonists of the proteins of the present invention. These antibodies can also be applied to antibody therapies for diseases related to the proteins of the present invention. When the obtained antibodies are administered to human bodies (antibody therapy), human antibodies or humanized antibodies are preferred due to their low immunogenicity.

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For example, transgenic animals comprising a repertoire of human antibody genes can be immunized with antigens selected from proteins, protein-expressing cells, or suspensions of the same. Antibody-producing cells are then recovered from the animals, fused with myeloma cells to yield hybridomas, and anti-protein human antibodies can be prepared from these hybridomas (*see* International Publication No. 92-03918, 94-02602, 94-25585, 96-33735, and 96-34096).

Alternatively, immunocytes such as immunized lymphocytes that produce antibodies, can be immortalized using cancer genes, and used to prepare monoclonal antibodies.

Monoclonal antibodies obtained in this way can be prepared using methods of genetic engineering (for example, *see* Borrebaeck, C.A.K. and Larrick, J.W., (1990) Therapeutic Monoclonal Antibodies, MacMillan Publishers, UK). For example, recombinant antibodies can be prepared by cloning DNAs that encode antibodies from immunocytes such as hybridomas or immunized lymphocytes that produce antibodies; then inserting these DNAs into appropriate vectors; and transforming these into host cells. Recombinant antibodies prepared as above can also be used in the present invention.

The antibodies can be modified by binding with a variety of molecules such as polyethylene glycols (PEGs). Antibodies modified in this way can also be used in the present invention. Modified antibodies can be obtained by chemically modifying antibodies. These kinds of modification methods are conventional to those skilled in the art. The antibodies can also be modified by other proteins. Antibodies modified by protein molecules can be produced using genetic engineering. That is, target proteins can be expressed by fusing antibody genes with genes that code for modification proteins.

Alternatively, such antibodies can be obtained as chimeric antibodies which comprise a non-human antibody-derived variable region and a human antibody-derived constant region, or as humanized antibodies which comprise a non-human antibody-derived complementarity determining region (CDR), a human antibody-derived framework region (FR), and a constant region. Such antibodies can be produced using known methods.

The standard techniques of molecular biology may be used to prepare DNA sequences coding for the chimeric and CDR-grafted products. Genes encoding the CDR of an antibody of interest are prepared, for example, by using the polymerase chain reaction (PCR) to synthesize the variable region from RNA of antibody-producing cells (*see*, for

example, Larrick *et al.*, "Methods: a Companion to Methods in Enzymology", vol. 2: page 106 (1991); Courtenay-Luck, "Genetic Manipulation of Monoclonal Antibodies" in Monoclonal Antibodies: Production, Engineering and Clinical Application; Ritter *et al.* (eds.), page 166 (Cambridge University Press, 1995), and Ward *et al.*, "Genetic Manipulation and
5 Expression of Antibodies" in Monoclonal Antibodies: Principles and Applications; Birch *et al.* (eds.), page 137 (Wiley-Liss, Inc., 1995)).

DNA sequences coding for the chimeric and CDR-grafted products may be synthesised completely or in part using oligonucleotide synthesis techniques. Site-directed mutagenesis and polymerase chain reaction techniques may be used as appropriate. For
10 example, oligonucleotide directed synthesis as described by Jones *et al.*, (1986) Nature.;321:522-5 may be used. Also oligonucleotide directed mutagenesis of a pre-existing variable region as, for example, described by Verhoeyen *et al.*, (1988) Science.;239:1534-6 or Riechmann *et al.*, (*supra*) may be used. Also enzymatic filling in of gapped oligonucleotides using T4 DNA polymerase as, for example, described by Queen *et al.*, (1989) Proc Natl Acad
15 Sci USA.;86:10029-33; PCT Publication WO 90/07861 may be used.

Any suitable host cell/vector system may be used for expression of the DNA sequences coding for the CDR-grafted heavy and light chains. Bacterial, *e.g.*, *E. coli*, and other microbial systems may be used, in particular for expression of antibody fragments such as FAb and (Fab')₂ fragments, and especially Fv fragments and single-chain antibody
20 fragments, *e.g.*, single-chain Fvs. Eucaryotic, *e.g.*, mammalian, host cell expression systems may be used, in particular, for production of larger CDR-grafted antibody products, including complete antibody molecules. Suitable mammalian host cells include CHO cells and myeloma or hybridoma cell lines.

Antibodies obtained as above can be purified until uniform. For example,
25 antibodies can be purified or separated according to general methods used for purifying and separating proteins. For example, antibodies can be separated and isolated using appropriately selected combinations of column chromatography, comprising but not limited to affinity chromatography, filtration, ultrafiltration, salt precipitation, dialysis, SDS polyacrylamide gel electrophoresis, isoelectric focusing, and so on (Antibodies : A
30 Laboratory Manual, Harlow and David, Lane (edit.), Cold Spring Harbor Laboratory, 1988).

Protein A columns and Protein G columns can be used as affinity columns. Exemplary protein A columns in use include Hyper D, POROS, and Sepharose FF

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(Pharmacia).

Exemplary chromatography (excluding affinity chromatography) include ion exchange chromatography, hydrophobic chromatography, gel filtration, reverse phase chromatography, and adsorption chromatography ("Strategies for Protein Purification and Characterization: A Laboratory Course Manual" Daniel R. Marshak *et al.*, (1996) Cold Spring Harbor Laboratory Press.). The chromatography can be performed according to the procedure of liquid phase chromatographies such as HPLC or FPLC.

For example, the antigen-binding activity of the antibodies of the present invention can be measured by using absorbance measurements, enzyme linked immunosorbent assays (ELISA), enzyme immunoassays (EIA), radioimmunoassays (RIA) and/or immunofluorescence methods. In ELISA, an antibody of the present invention is immobilized on a plate, a protein of the present invention is added to the plate, and then a sample comprising the desired antibody such as the culture supernatant of cells that produce the antibody or purified antibody is added. A secondary antibody that recognizes the primary antibody and has been tagged with an enzyme such as alkaline phosphatase is then added, and the plate is incubated. After washing, an enzyme substrate such as p-nitrophenyl phosphate is added to the plate, absorbance is measured, and the antigen-binding activity of the samples is evaluated. Protein fragments (C-terminal or N-terminal fragments, and such) can be used in the same way as proteins. The binding activity of the antibodies can be evaluated using BIAcore (Pharmacia).

Furthermore, one or more anti-REG4 antibodies which inhibit REG4 activity are used for the methods and compositions of the present invention. In the present invention, a preferable anti-REG4 antibody neutralizes REG4 activity to promote cell proliferation of pancreatic cancer. The neutralizing function of anti-REG4 antibody can be evaluated *in vitro* or *in vivo*. For instance, the neutralizing function of anti-REG4 antibody can be estimated by observing the effect of the antibody on proliferation of REG4 expressing cells. Specifically, the neutralizing function would be acknowledged, when the antibody detectably suppresses the cell proliferation as measured using any method known in the art. Alternatively, such function also be confirmed by inhibition of Akt signaling pathway (Sekikawa A, *et al.* Gastroenterology 2005; 128: 642-53., Bishnupuri KS, *et al.* Gastroenterology 2006; 130: 137-49.).

In the present invention, anti-REG4 antibodies and non-antibody binding proteins

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can be administered to humans or other animals as pharmaceutical agents. In the present invention, animals other than humans to which the antibodies can be administered include mice, rats, guinea pigs, rabbits, chickens, cats, dogs, sheep, pigs, cows, monkeys, baboons, and chimpanzees. The antibodies and non-antibody binding proteins can be directly
5 administered to subjects, and in addition, can be formulated into dosage forms using known pharmaceutical formulation methods. For example, depending on requirements, they can be parenterally administered in an injectable form such as a sterile solution or suspension with water or other arbitrary pharmaceutically acceptable fluid. For example, this kind of compounds can be mixed with acceptable carriers or solvents, specifically sterile water,
10 physiological saline, vegetable oils, emulsifiers, suspension agents, surfactants, stabilizers, flavoring agents, excipients, solvents, preservatives, binding agents and the like, into a generally accepted unit dosage essential for use as a pharmaceutical agent.

Other isotonic solutions comprising physiological saline, glucose, and adjuvants (such as D-sorbitol, D-mannose, D-mannitol, and sodium chloride) can be used as the
15 injectable aqueous solution. They can also be used with appropriate solubilizers such as alcohols, specifically ethanols and polyalcohols (for example, propylene glycols and polyethylene glycol), and non-ionic surfactants (for example Polysorbate 80TM or HCO-50).

Sesame oils or soybean oils can be used as an oleaginous solution, and benzyl benzoate or benzyl alcohols can be used with them as a solubilizer. Buffer solutions
20 (phosphate buffers, sodium acetate buffers, or so on), analgesics (procaine hydrochloride or such), stabilizers (benzyl alcohol, phenols, or so on), and antioxidants can be used in the formulation. The prepared injections can be packaged into appropriate ampules.

In the present invention, the anti-REG4 antibodies and non-antibody binding proteins can be administered to patients, for example, intraarterially, intravenously, percutaneously,
25 intranasally, transbronchially, locally, or intramuscularly. Intravascular (intravenous) administration by drip or injection is an example of a general method for systematic administration of antibodies to pancreatic cancer patients. In addition, methods in which an intraarterial catheter is inserted near a vein that supplies nutrients to cancer cells to locally inject anti-cancer agents such as antibody agents are effective as local control therapies for
30 metastatic focuses as well as primary focuses of pancreatic cancer.

Although dosage and administration methods vary according to patient body weight and age, and administration method, these can be routinely selected by one skilled in

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the art. In addition, DNA encoding an antibody can be inserted into a vector for gene therapy, and the vector can be administered for therapy. Dosage and administration methods vary according to patient body weight, age, and condition, however, one skilled in the art can select these appropriately. Usually a lower dose is administered at first and then
5 incrementally increased until an efficacious effect is achieved without undesirable side effects.

Anti-REG4 antibodies and non-antibody binding proteins can be administered to living bodies in an amount such that neutralizing function against REG4 can be confirmed. For example, although there is a certain amount of difference depending on symptoms, anti-REG4 antibody dosage is 0.1 mg to 250 mg/kg per day. Usually, the dosage for an adult (of
10 weight 60 kg) is 5 mg to 17.5 g/day, preferably 5 mg to 10 g/day, and more preferably 100 mg to 3 g/day. The dosage schedule is from one to ten times over a two to ten day interval, and for example, progress is observed after a three to six times administration.

Alternatively, nucleic acids comprising sequences encoding antibodies, non-antibody binding proteins, or functional derivatives thereof, are administered to treat or
15 prevent diseases associated with REG4-expressing cells, such as pancreatic cancer including PDAC, by way of gene therapy. Gene therapy refers to therapy performed by the administration to a subject of an expressed or expressible nucleic acid. In this embodiment of the invention, the nucleic acids produce their encoded antibody or antibody fragment that mediates a prophylactic or therapeutic effect.

20 Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, *see* Goldspiel *et al.*, (1993) Clin. Pharm.;12:488-505; Wu and Wu, (1991) Biotherapy.;3:87-95; Tolstoshev, (1993) Ann Rev Pharmacol Toxicol.;32:573-96; Mulligan, (1993) Science.;260:926-32; Morgan and Anderson,
25 (1993) Ann Rev Biochem.;62:191-217; Trends Biotechnol.;11(5):155-215. Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel *et al.* (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, NY (1993); Kriegler, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY (1990).

30 In a preferred aspect, a composition of the invention comprises nucleic acids encoding an antibody, or a non-antibody binding protein, said nucleic acids being part of an expression vector that expresses the antibody or fragments or chimeric proteins or heavy or light chains

thereof in a suitable host. In particular, such nucleic acids have promoters, preferably heterologous promoters, operably linked to the antibody coding region, said promoter being inducible or constitutive, and, optionally, tissue-specific. In another particular embodiment, nucleic acid molecules are used in which the antibody coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the antibody encoding nucleic acids (Koller and Smithies, (1989) Proc Natl Acad Sci USA.;86:8932-5; Zijlstra *et al.*, (1989) Nature.;342:435-8). In specific embodiments, the expressed antibody molecule is a single chain antibody; alternatively, the nucleic acid sequences include sequences encoding both the heavy and light chains, or fragments thereof, of the antibody.

Delivery of the nucleic acids into a subject may be either direct, in which case the subject is directly exposed to the nucleic acid or nucleic acid-carrying vectors, or indirect, in which case, cells are first transformed with the nucleic acids *in vitro*, then transplanted into the subject. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene therapy.

In a specific embodiment, the nucleic acid sequences are directly administered *in vivo*, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, *e.g.*, by constructing them as part of an appropriate nucleic acid expression vector and administering it so that they become intracellular, *e.g.*, by infection using defective or attenuated retrovirals or other viral vectors (*see* U.S. Pat. No. 4,980,286), or by direct injection of naked DNA, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, encapsulation in liposomes, microparticles, or microcapsules, or by administering them in linkage to a peptide which is known to enter the nucleus, by administering it in linkage to a ligand subject to receptor-mediated endocytosis (*see, e.g.*, Wu and Wu, (1987) J Biol Chem.;262:4429-32) (which can be used to target cell types specifically expressing the receptors), *etc.*

In another embodiment, nucleic acid-ligand complexes can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (*see, e.g.*, PCT Publications WO 92/06180, WO 92/22635, WO92/20316, WO93/14188 or WO 93/20221).

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Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, (1989) Proc Natl Acad Sci USA.;86:8932-5; Zijlstra *et al.*, (1989) Nature.;342:435-8).

In a specific embodiment, viral vectors that contains nucleic acid sequences encoding an antibody of the invention or fragments thereof are used. For example, a retroviral vector can be used (*see* Miller *et al.*, (1993) Methods Enzymol.;217:581-99). These retroviral vectors contain the components necessary for the correct packaging of the viral genome and integration into the host cell DNA. The nucleic acid sequences encoding the antibody or non-antibody binding proteins to be used in gene therapy are cloned into one or more vectors, which facilitates delivery of the gene into a subject. More detail about retroviral vectors can be found in Boesen *et al.*, (1994) Biotherapy.;6:291-302, which describes the use of a retroviral vector to deliver the *mdr 1* gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes *et al.*, (1994) J Clin Invest.;93:644-51; Keim *et al.*, (1994) Blood.;83:1467-73; Salmons and Gunzberg, (1993) Hum Gene Ther.;4:129-41; Grossman and Wilson, (1993) Curr Opin Genet Dev.;3:110-4.

Adenoviruses are other viral vectors that can be used in gene therapy. Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson, in (1993) Curr Opin Genet Dev.;3:499-503, present a review of adenovirus-based gene therapy. Bout *et al.*, in (1994) Hum Gene Ther.;5:3-10, demonstrates the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld *et al.*, (1991) Science.;252:431-4; Rosenfeld *et al.*, (1992) Cell.;68:143-55; Mastrangeli *et al.*, (1993) J Clin Invest.;91:225-34; PCT Publication WO94/12649; Wang *et al.*, (1995) Gene Ther.;2:775-83. In a preferred embodiment, adenovirus vectors are used.

Adeno-associated virus (AAV) has also been proposed for use in gene therapy (Walsh *et al.*, (1993) Proc Soc Exp Biol Med.;204:289-300; U.S. Pat. No. 5,436,146).

Another approach to gene therapy involves transferring a gene to cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or

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viral infection. Usually, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a subject.

In this embodiment, the nucleic acid is introduced into a cell prior to administration
5 *in vivo* of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcellmediated gene transfer, spheroplast fusion, *etc.* Numerous techniques are known in the art for the introduction of
10 foreign genes into cells (*see, e.g.*, Loeffler and Behr, (1993) *Methods Enzymol.*;217:599-618; Cotton *et al.*, 1993, *Methods Enzymol.*;217:618-44; Cline MJ. *Pharmacol Ther.* 1985;29(1):69-92.) and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell,
15 so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny.

The resulting recombinant cells can be delivered to a subject by various methods known in the art. Recombinant blood cells (*e.g.*, hematopoietic stem or progenitor cells) are preferably administered intravenously. The amount of cells envisioned for use depends on
20 the desired effect, patient state, *etc.*, and can be determined by one skilled in the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited to epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils,
25 megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, *e.g.*, as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, *etc.* In a preferred embodiment, the cell used for gene therapy is autologous to the subject.

In an embodiment in which recombinant cells are used in gene therapy, nucleic
30 acid sequences encoding an antibody or fragment thereof are introduced into the cells such that they are expressible by the cells or their progeny, and the recombinant cells are then administered *in vivo* for therapeutic effect. In a specific embodiment, stem or progenitor

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cells are used. Any stem and/or progenitor cells which can be isolated and maintained *in vitro* can potentially be used in accordance with this embodiment of the present invention (*see e.g.*, PCT Publication WO 94/08598; Stemple and Anderson, (1992) *Cell*;71:973-85; Rheinwald. (1980) *Methods Cell Biol.*;21A:229-54; Pittelkow and Scott, (1986) *Mayo Clin Proc.*;61:771-7).

In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding region, such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription.

In addition, the present invention provides immunogenic compositions for inducing antibodies comprising neutralizing functions against REG4, where the compositions comprise as an active ingredient REG4 or an immunologically active REG4 fragment, or a DNA or cell which can express the same. Alternatively, the present invention relates to uses of REG4 or an immunologically active REG4 fragment, or a DNA or cell which can express the same in the production of immunogenic compositions for inducing antibodies comprising neutralizing functions against REG4.

In the present invention, neutralizing of REG4 activity to promote cell proliferation in autocrine/paracrine manner can be achieved by the administration of anti-REG4 antibodies. Thus, if REG4 antibodies can be induced *in vivo*, therapeutic effects equivalent to the antibody administration can be achieved. When administering immunogenic compositions comprising antigens, target antibodies can be induced *in vivo*. The immunogenic compositions of the present invention thus are particularly useful in vaccine therapy against REG4-expressing cells. Thus, the immunogenic compositions of the present invention are effective as, for example, vaccine compositions for pancreatic cancer therapies.

The immunogenic compositions of the present invention can comprise REG4 or an immunologically active REG4 fragment, as an active ingredient. An immunologically active REG4 fragment refers to a fragment that can induce anti- REG4 antibodies which recognize REG4 and comprise neutralizing function. Below, REG4 and the immunologically active REG4 fragment are described as immunogenic proteins. Whether a given fragment induces target antibodies can be determined by actually immunizing an animal, and confirming the activity of the induced antibodies. Antibody induction and the confirmation of its activity can be carried out, for example, using methods described in Examples.

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The immunogenic compositions of the present invention comprise pharmaceutically acceptable carriers as well as immunogenic proteins, the active ingredients. If necessary, the compositions can also be combined with an adjuvant. Killed tuberculosis bacteria, diphtheria toxoid, saponin and so on can be used as the adjuvant.

5 Alternatively, DNAs coding for the immunogenic proteins, or cells retaining those DNAs in an expressible state, can be used as the immunogenic compositions. Methods for using DNAs expressing the target antigen as immunogens, so-called DNA vaccines, are well known. DNA vaccines can be obtained by inserting a DNA encoding REG4 or its fragment into an appropriate expression vector.

10 Retrovirus vectors, adenovirus vectors, adeno-associated virus vectors, Sendai virus vectors or such can be used as the vector. In addition, DNAs in which a DNA encoding an immunogenic protein is functionally connected downstream of a promoter can be directly introduced into cells as naked DNA, and then expressed. Naked DNA can be encapsulated in liposomes or viral envelope vectors and introduced into cells.

15 As noted above, the present invention provides methods for inducing antibodies which comprise neutralizing function against REG4, where the methods comprise the step of administering REG4, an immunologically active REG4 fragment, or DNA or cells that can express the same. The methods of the present invention induce antibodies that comprise neutralizing function that suppresses cell growth of REG4-expressing cells such as pancreatic
20 cancers. As a result, therapeutic effects for pancreatic cancers and so on can be obtained.

Dominant negative protein that inhibits KIAA0101:

The present invention relates to inhibitory polypeptides that contain QKGIGEFF (SEQ ID NO: 46). In some preferred embodiments, the inhibitory polypeptide comprises QKGIGEFF (SEQ ID NO: 46); a polypeptide functionally equivalent to the polypeptide; or
25 polynucleotide encoding those polypeptides, wherein the polypeptide lacks the biological function of a peptide consisting of SEQ ID NO: 40. The amino acid sequence set forth in SEQ ID NO: 40 is disclosed in WO2004/31412. It has been known that cancer cell proliferation can be controlled by inhibiting the expression of the amino acid sequence. However, it is a novel finding proved by the present inventors that a fragment containing a
30 sequence with a specific mutation in the above amino acid sequence inhibits the cancer cell proliferation.

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The polypeptides comprising the selected amino acid sequence of the present invention, can be of any length, so long as the polypeptide inhibits cancer cell proliferation. Specifically, the length of the amino acid sequence may range from 8 to 70 residues, for example, from 8 to 50, preferably from 8 to 30, more specifically from 8 to 20, further more specifically from 8 to 16 residues. For example, the amino acid sequence
5 VRPTPKWQKGIGEFFRLSPK/SEQ ID NO. 44 is preferable as the above-described selected amino acid sequence. Therefore, a polypeptide comprising or consisting of the amino acid sequence TPKWQKGIGEFFRLSP/SEQ ID NO. 45 is a preferred example of the polypeptides in the present invention. The polypeptides of the present invention, which are
10 characterized by containing the amino acid sequence QKGIGEFF/SEQ ID NO: 46, may also be referred to as "PIP binding motif (PIP box)".

The polypeptides of the present invention may contain two or more "selected amino acid sequences". The two or more "selected amino acid sequences" may be the same or different amino acid sequences. Furthermore, the "selected amino acid sequences" can be
15 linked directly. Alternatively, they may be disposed with any intervening sequences among them.

Furthermore, the present invention relates to polypeptides homologous (*i.e.*, share sequence identity) to the QKGIGEFF/SEQ ID NO: 46 polypeptide specifically disclosed here. In the present invention, polypeptides homologous to the QKGIGEFF/SEQ ID NO: 46
20 polypeptide are those which contain any mutations selected from addition, deletion, substitution and insertion of one or several amino acid residues and are functionally equivalent to the QKGIGEFF/SEQ ID NO: 46 polypeptide. The phrase "functionally equivalent to the QKGIGEFF/SEQ ID NO: 46 polypeptide" refers to having the function to inhibit the binding of KIAA0101 to PCNA. The QKGIGEFF/SEQ ID NO: 46 sequence is
25 preferably conserved in the amino acid sequences constituting polypeptides functionally equivalent to QKGIGEFF/SEQ ID NO: 46 polypeptide. Therefore, polypeptides functionally equivalent to the QKGIGEFF/SEQ ID NO: 46 peptide in the present invention preferably have amino acid mutations in sites other than the QKGIGEFF/SEQ ID NO: 46 sequence. Amino acid sequences of polypeptides functionally equivalent to the
30 QKGIGEFF/SEQ ID NO: 46 peptide in the present invention conserve the QKGIGEFF/SEQ ID NO: 46 sequence, and have 60% or higher, usually 70% or higher, preferably 80% or higher, more preferably 90% or higher, or 95% or higher, and further more preferably 98% or

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higher homology to a "selected amino acid sequence". Amino acid sequence homology can be determined using algorithms well known in the art, for example, BLAST or ALIGN set to their default settings.

Alternatively, the number of amino acids that may be mutated is not particularly restricted, so long as the QKGIGEFF/SEQ ID NO: 46 peptide activity is maintained. Generally, up to about 50 amino acids may be mutated, preferably up to about 30 amino acids, more preferably up to about 10 amino acids, and even more preferably up to about 3 amino acids. Likewise, the site of mutation is not particularly restricted, so long as the mutation does not result in the disruption of the QKGIGEFF/SEQ ID NO: 46 peptide activity.

In a preferred embodiment, the activity of the QKGIGEFF/SEQ ID NO: 46 peptide comprises apoptosis inducing effect in a KIAA0101 expressing cell, i.e. pancreatic cancer cell, prostatic cancer cell, breast cancer cell, and bladder cancer cell. Apoptosis means cell death caused by the cell itself and is sometimes referred to as programmed cell death. Aggregation of nuclear chromosome, fragmentation of nucleus, or condensation of cytoplasm is observed in a cell undergoing apoptosis. Methods for detecting apoptosis are well known. For instance, apoptosis may be confirmed by TUNEL staining (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling; Gavrieli et al., (1992) J. Cell Biol. 119: 493-501, Mori et al., (1994) Anat. & Embryol. 190: 21-28). Alternatively, DNA ladder assays, Annexin V staining, caspase assay, electron microscopy, or observation of conformational alterations on nucleus or cell membrane may be used for detecting apoptosis. Any commercially available kits may be used for detecting these behaviors in cells which are induced by apoptosis. For example, such apoptosis detection kits may be commercially available from the following providers:

LabChem Inc.,
Promega,
BD Biosciences Pharmingen,
Calbiochem,
Takara Bio Company (CLONTECH Inc.),
CHEMICON International, Inc,
Medical & Biological Laboratories Co., Ltd. etc.

The polypeptides of the present invention can be chemically synthesized from any position based on selected amino acid sequences. Methods used in the ordinary peptide

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chemistry can be used for the method of synthesizing polypeptides. Specifically, the methods include those described in the following documents and Japanese Patent publications:

Peptide Synthesis, Interscience, New York, 1966; The Proteins, Vol. 2, Academic Press Inc., New York, 1976;

Peputido gousei (Peptide Synthesis), Maruzen (Inc.), 1975;

Peputido gousei no kiso to jikken (Fundamental and Experimental Peptide Synthesis), Maruzen (Inc.), 1985;

Iyaku hin no kaihatsu (Development of Pharmaceuticals), Sequel, Vol. 14: Peputido gousei (Peptide Synthesis), Hirokawa Shoten, 1991;

International Patent Publication WO99/67288.

The polypeptides of the present invention can be also synthesized by known genetic engineering techniques. An example of genetic engineering techniques is as follows. Specifically, DNA encoding a desired peptide is introduced into an appropriate host cell to prepare a transformed cell. The polypeptides of the present invention can be obtained by recovering polypeptides produced by this transformed cell. Alternatively, a desired polypeptide can be synthesized with an in vitro translation system, in which necessary elements for protein synthesis are reconstituted in vitro.

When genetic engineering techniques are used, the polypeptide of the present invention can be expressed as a fused protein with a peptide having a different amino acid sequence. A vector expressing a desired fusion protein can be obtained by linking a polynucleotide encoding the polypeptide of the present invention to a polynucleotide encoding a different peptide so that they are in the same reading frame, and then introducing the resulting nucleotide into an expression vector. The fusion protein is expressed by transforming an appropriate host with the resulting vector. Different peptides to be used in forming fusion proteins include the following peptides:

FLAG (Hopp et al., (1988) BioTechnology 6, 1204-10),

6xHis consisting of six His (histidine) residues, 10xHis,

Influenza hemagglutinin (HA),

Human c-myc fragment,

VSV-GP fragment,

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p18 HIV fragment,
T7-tag,
HSV-tag,
E-tag,
5 SV40T antigen fragment,
Ick tag,
 α -tubulin fragment,
B-tag,
Protein C fragment,
10 GST (glutathione-S-transferase),
HA (Influenza hemagglutinin),
Immunoglobulin constant region,
 β -galactosidase, and
MBP (maltose-binding protein).

15 The polypeptide of the present invention can be obtained by treating the fusion protein thus produced with an appropriate protease, and then recovering the desired polypeptide. To purify the polypeptide, the fusion protein is captured in advance with affinity chromatography that binds with the fusion protein, and then the captured fusion protein can be treated with a protease. With the protease treatment, the desired polypeptide
20 is separated from affinity chromatography, and the desired polypeptide with high purity is recovered.

The polypeptides of the present invention include modified polypeptides. In the present invention, the term "modified" refers, for example, to binding with other substances. Accordingly, in the present invention, the polypeptides of the present invention may further
25 comprise other substances such as cell-membrane permeable substance. The other substances include organic compounds such as peptides, lipids, saccharides, and various naturally-occurring or synthetic polymers. The polypeptides of the present invention may have any modifications so long as the polypeptides retain the desired activity of inhibiting the binding of KIAA0101 to PCNA. In some embodiments, the inhibitory polypeptides can
30 directly compete with KIAA0101 binding to PCNA. Modifications can also confer additive functions on the polypeptides of the invention. Examples of the additive functions include targetability, deliverability, and stabilization.

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Preferred examples of modifications in the present invention include, for example, the introduction of a cell-membrane permeable substance. Usually, the intracellular structure is cut off from the outside by the cell membrane. Therefore, it is difficult to efficiently introduce an extracellular substance into cells. Cell membrane permeability can be conferred on the polypeptides of the present invention by modifying the polypeptides with a cell-membrane permeable substance. As a result, by contacting the polypeptide of the present invention with a cell, the polypeptide can be delivered into the cell to act thereon.

The "cell-membrane permeable substance" refers to a substance capable of penetrating the mammalian cell membrane to enter the cytoplasm. For example, a certain liposome fuses with the cell membrane to release the content into the cell. Meanwhile, a certain type of polypeptide penetrates the cytoplasmic membrane of mammalian cell to enter the inside of the cell. For polypeptides having such a cell-entering activity, cytoplasmic membranes and such in the present invention are preferable as the substance. Specifically, the present invention includes polypeptides having the following general formula.

[R]-[D];

wherein,

[R] represents a cell-membrane permeable substance; [D] represents a fragment sequence containing QKGIGEFF/SEQ ID NO: 46. In the above-described general formula, [R] and [D] can be linked directly or indirectly through a linker. Peptides, compounds having multiple functional groups, or such can be used as a linker. Specifically, amino acid sequences containing -GGG- can be used as a linker. Alternatively, a cell-membrane permeable substance and a polypeptide containing a selected sequence can be bound to the surface of a minute particle. [R] can be linked to any positions of [D]. Specifically, [R] can be linked to the N terminal or C terminal of [D], or to a side chain of amino acids constituting [D]. Furthermore, more than one [R] molecule can be linked to one molecule of [D]. The [R] molecules can be introduced to different positions on the [D] molecule. Alternatively, [D] can be modified with a number of [R]s linked together.

For example, there have been reported a variety of naturally-occurring or artificially synthesized polypeptides having cell-membrane permeability (Joliot A. & Prochiantz A., Nat Cell Biol. 2004; 6: 189-96). All of these known cell-membrane permeable substances can be used for modifying polypeptides in the present invention. In the present invention, for example, any substance selected from the following group can be used as the above-described

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cell-permeable substance:

poly-arginine; Matsushita et al., (2003) J. Neurosci.; 21, 6000-7.

[Tat / RKKRRQRRR] (SEQ ID NO: 47) Frankel et al., (1988) Cell 55, 1189-93.

Green & Loewenstein (1988) Cell 55, 1179-88.

5 [Penetratin / RQIKIWFQNRRMKWKK] (SEQ ID NO: 48)

Derossi et al., (1994) J. Biol. Chem. 269, 10444-50.

[Buforin II / TRSSRAGLQFPVGRVHRLLRK] (SEQ ID NO: 49)

Park et al., (2000) Proc. Natl Acad. Sci. USA 97, 8245-50.

[Transportan / GWTLNSAGYLLGKINLKALAALAKKIL] (SEQ ID NO: 50)

10 Pooga et al., (1998) FASEB J. 12, 67-77.

[MAP (model amphipathic peptide) / KLALKLALKALKAAALKLA] (SEQ ID NO: 51)

Oehlke et al., (1998) Biochim. Biophys. Acta. 1414, 127-39.

[K-FGF / AAVALLPAVLLALLAP] (SEQ ID NO: 52)

15 Lin et al., (1995) J. Biol. Chem. 270, 14255-8.

[Ku70 / VPMLK] (SEQ ID NO: 53)

Sawada et al., (2003) Nature Cell Biol. 5, 352-7.

[Ku70 / PMLKE] (SEQ ID NO: 61)

Sawada et al., (2003) Nature Cell Biol. 5, 352-7.

20 [Prion / MANLGYWLLALFVTMTDVGGLCKKRPKP] (SEQ ID NO: 54)

Lundberg et al., (2002) Biochem. Biophys. Res. Commun. 299, 85-90.

[pVEC / LLILRRRIRKQAAHAHSK] (SEQ ID NO: 55)

Elmquist et al., (2001) Exp. Cell Res. 269, 237-44.

[Pep-1 / KETWWETWWTEWSQPKKKRKV] (SEQ ID NO: 56)

25 Morris et al., (2001) Nature Biotechnol. 19, 1173-6.

[SynB1 / RGGRLSYSRRRFSTSTGR] (SEQ ID NO: 57)

Rousselle et al., (2000) Mol. Pharmacol. 57, 679-86.

[Pep-7 / SDLWEMMMVSLACQY] (SEQ ID NO: 58)

Gao et al., (2002) Bioorg. Med. Chem. 10, 4057-65.

30 [HN-1 / TSPLNIHNGQKL] (SEQ ID NO: 59)

Hong & Clayman (2000) Cancer Res. 60, 6551-6.

In the present invention, the poly-arginine, which is listed above as an example of cell-

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membrane permeable substances, is constituted by any number of arginine residues. Specifically, for example, it is constituted by consecutive 5-20 arginine residues. The preferable number of arginine residues is 11 (SEQ ID NO: 60).

Pharmaceutical compositions comprising QKGIGEFF/SEQ ID NO: 46

5 The polypeptides of the present invention inhibit proliferation of cancer cells. Therefore, the present invention provides therapeutic and/or preventive agents for cancer which comprise as an active ingredient a polypeptide which comprises QKGIGEFF/SEQ ID NO: 46; or a polynucleotide encoding the same. Alternatively, the present invention relates to methods for treating and/or preventing cancer comprising the step of administering a
10 polypeptide of the present invention. Furthermore, the present invention relates to the use of the polypeptides of the present invention in manufacturing pharmaceutical compositions for treating and/or preventing cancer. Cancers which can be treated or prevented by the present invention are not limited, so long as expression of KIAA0101 is up-regulated in the cancer cells. For example, the polypeptides of the present invention are useful for treating
15 pancreatic cancer, lung cancer, prostatic cancer, breast cancer, bladder cancer, kidney cancer or testicular tumors. Among them, pancreatic cancer is particularly preferable as a target for treatment or prevention in the present invention.

 Alternatively, the inhibitory polypeptides of the present invention can be used to induce apoptosis of cancer cells. Therefore, the present invention provides apoptosis
20 inducing agents for cells, which comprise as an active ingredient a polypeptide which comprises QKGIGEFF/SEQ ID NO: 46; or a polynucleotide encoding the same. The apoptosis inducing agents of the present invention may be used for treating cell proliferative diseases such as cancer. Cancers which can be treated or prevented by the present invention are not limited, so long as expression of KIAA0101 is up-regulated in the cancer cells. For
25 example, the polypeptides of the present invention are useful in treating pancreatic cancer, prostatic cancer, breast cancer, bladder cancer, lung cancer, kidney cancer or testicular tumors. Among them, pancreatic cancer is particularly preferable as a target for treatment or prevention in the present invention. Alternatively, the present invention relates to methods for inducing apoptosis of cells which comprise the step of administering the polypeptides of
30 the present invention. Furthermore, the present invention relates to the use of polypeptides of the present invention in manufacturing pharmaceutical compositions for inducing apoptosis in cells.

The inhibitory polypeptides of the present invention induce apoptosis in KIAA0101-expressing cells such as pancreatic cancer. In the meantime, KIAA0101 expression has not been observed in most of normal organs. In some normal organs, the expression level of KIAA0101 is relatively low as compared with cancer tissues.

5 Accordingly, the polypeptides of the present invention may induce apoptosis specifically in cancer cells.

When the polypeptides of the present invention are administered, as a prepared pharmaceutical, to human and other mammals such as mouse, rat, guinea pig, rabbit, cat, dog, sheep, pig, cattle, monkey, baboon and chimpanzee for treating cancer or inducing apoptosis
10 in cells, isolated compounds can be administered directly, or formulated into an appropriate dosage form using known methods for preparing pharmaceuticals. For example, if necessary, the pharmaceuticals can be orally administered as a sugar-coated tablet, capsule, elixir, and microcapsule, or alternatively parenterally administered in the injection form that is a sterilized solution or suspension with water or any other pharmaceutically acceptable liquid.
15 For example, the compounds can be mixed with pharmacologically acceptable carriers or media, specifically sterilized water, physiological saline, plant oil, emulsifier, suspending agent, surfactant, stabilizer, corrigent, excipient, vehicle, preservative, and binder, in a unit dosage form necessary for producing a generally accepted pharmaceutical. Depending on the amount of active ingredient in these formulations, a suitable dose within the specified
20 range can be determined.

Examples of additives that can be mixed in tablets and capsules are binders such as gelatin, corn starch, tragacanth gum, and gum arabic; media such as crystalline cellulose; swelling agents such as corn starch, gelatin, and alginic acid; lubricants such as magnesium stearate; sweetening agents such as sucrose, lactose or saccharine; and corrigents such as
25 peppermint, wintergreen oil and cherry. When the unit dosage form is capsule, liquid carriers such as oil can be further included in the above-described ingredients. Sterilized mixture for injection can be formulated using media such as distilled water for injection according to the realization of usual pharmaceuticals.

Physiological saline, glucose, and other isotonic solutions containing adjuvants such
30 as D-sorbitol, D-mannose, D-mannitol, and sodium chloride can be used as an aqueous solution for injection. They can be used in combination with a suitable solubilizer, for example, alcohol, specifically ethanol and polyalcohols such as propylene glycol and

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polyethylene glycol, non-ionic surfactants such as Polysorbate 80™ and HCO-50.

Sesame oil or soybean oil can be used as an oleaginous liquid, and also used in combination with benzyl benzoate or benzyl alcohol as a solubilizer. Furthermore, they can be further formulated with buffers such as phosphate buffer and sodium acetate buffer; analgesics such as procaine hydrochloride; stabilizers such as benzyl alcohol and phenol; and antioxidants. Injections thus prepared can be loaded into appropriate ampoules.

Methods well-known to those skilled in the art can be used for administering pharmaceutical compounds of the present invention to patients, for example, by intraarterial, intravenous, or subcutaneous injection, and similarly, by intranasal, transtracheal, intramuscular, or oral administration. Doses and administration methods are varied depending on the body weight and age of patients as well as administration methods. However, those skilled in the art can routinely select them. DNA encoding a polypeptide of the present invention can be inserted into a vector for the gene therapy, and the vector can be administered for treatment. Although doses and administration methods are varied depending on the body weight, age, and symptoms of patients, those skilled in the art can appropriately select them. For example, a dose of the compound which bind to the polypeptides of the present invention so as to regulate their activity is, when orally administered to a normal adult (body weight 60 kg), about 0.1 mg to about 100 mg/day, preferably about 1.0 mg to about 50 mg/day, more preferably about 1.0 mg to about 20 mg/day, although it is slightly varied depending on symptoms.

When the compound is parenterally administered to a normal adult (body weight 60 kg) in the injection form, it is convenient to intravenously inject a dose of about 0.01 mg to about 30 mg/day, preferably about 0.1 mg to about 20 mg/day, more preferably about 0.1 mg to about 10 mg/day, although it is slightly varied depending on patients, target organs, symptoms, and administration methods. Similarly, the compound can be administered to other animals in an amount converted from the dose for the body weight of 60 kg.

Pharmaceutical compositions:

Accordingly, the present invention includes medicaments and methods useful in either or both preventing and treating cancers. These medicaments and methods comprise at least a siRNA that inhibits expression of REG4 or KIAA0101, the antibody that neutralizes the activity of REG4, a polypeptide which comprises QKGIGEFF/SEQ ID NO: 46; a polypeptide functionally equivalent to the polypeptide; or polynucleotide encoding those polypeptides of

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the present invention in an amount effective to achieve attenuation or arrest of disease cell proliferation. More specifically, in the context of the present invention, a therapeutically effective amount means an amount effective to prevent development of, or to alleviate existing symptoms of, the subject being treated.

5 Individuals to be treated with methods of the present invention include any individual afflicted with cancer, including, *e.g.*, pancreatic cancer, prostatic cancer, breast cancer, and bladder cancer. Such an individual can be, for example, a vertebrate such as a mammal, including a human, dog, cat, horse, cow, or goat; or any other animal, particularly a commercially important animal or a domesticated animal. For purposes of the present
10 invention, elevated expression of marker proteins refers to a mean cellular marker protein concentration for one or both marker proteins that is at least 10%, preferably 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55% or more above normal mean cellular concentration of the marker protein(s).

 In the context of the present invention, suitable pharmaceutical formulations include
15 those suitable for oral, rectal, nasal, topical (including buccal and sub-lingual), vaginal or parenteral (including intramuscular, sub-cutaneous and intravenous) administration, or for administration by inhalation or insufflation. Preferably, administration is intravenous. The formulations are optionally packaged in discrete dosage units.

 Pharmaceutical formulations suitable for oral administration include capsules, cachets
20 or tablets, each containing a predetermined amount of active ingredient. Suitable formulations also include powders, granules, solutions, suspensions and emulsions. The active ingredient is optionally administered as a bolus electuary or paste. Tablets and capsules for oral administration may contain conventional excipients, for example, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol, cellulose preparations such as
25 maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP), binding agents, lubricants, and/or wetting agents. If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

30 A tablet may be made by compression or molding, optionally with one or more formulation ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredients in a free-flowing form, such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, lubricating, surface active and/or

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dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may be coated according to methods well known in the art. Oral fluid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or
5 may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may contain conventional additives such as suspending agents, emulsifying agents, non-aqueous vehicles (which may include edible oils), pH maintaining agents, and/or preservatives. The tablets may optionally be formulated so as to provide slow or controlled release of the active ingredient therein. A package of tablets may contain one
10 tablet to be taken on each of the month.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions, optionally contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; as well as aqueous and non-aqueous sterile suspensions including suspending agents and/or thickening
15 agents. The formulations may be presented in unit dose or multi-dose containers, for example as sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, saline, water-for-injection, immediately prior to use. Alternatively, the formulations may be presented for continuous infusion. Extemporaneous injection solutions and suspensions may be prepared
20 from sterile powders, granules and tablets of the kind previously described.

Formulations suitable for rectal administration include suppositories with standard carriers such as cocoa butter or polyethylene glycol. Formulations suitable for topical administration in the mouth, for example buccally or sublingually, include lozenges, containing the active ingredient in a flavored base such as sucrose and acacia or tragacanth,
25 and pastilles comprising the active ingredient in a base such as gelatin and glycerin or sucrose and acacia. For intra-nasal administration the compounds of the invention may be used as a liquid spray, a dispersible powder or in the form of drops. Drops may be formulated with an aqueous or non-aqueous base also comprising one or more dispersing agents, solubilizing agents and/or suspending agents.

30 For administration by inhalation the compounds can be conveniently delivered from an insufflator, nebulizer, pressurized packs or other convenient means of delivering an aerosol spray. Pressurized packs may comprise a suitable propellant such as

dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount.

Alternatively, for administration by inhalation or insufflation, the compounds may
5 take the form of a dry powder composition, for example a powder mix of the compound and a suitable powder base such as lactose or starch. The powder composition may be presented in unit dosage form, for example, as capsules, cartridges, gelatin or blister packs from which the powder may be administered with the aid of an inhalator or insufflators.

Other formulations include implantable devices and adhesive patches; which release
10 a therapeutic agent.

When desired, the above described formulations, adapted to give sustained release of the active ingredient, may be employed. The pharmaceutical compositions may also contain other active ingredients such as antimicrobial agents, immunosuppressants and/or preservatives.

15 It should be understood that in addition to the ingredients particularly mentioned above, the formulations of this invention may include other agents conventional in the art with regard to the type of formulation in question. For example, formulations suitable for oral administration may include flavoring agents.

It will be apparent to those persons skilled in the art that certain excipients may be
20 more preferable depending upon, for instance, the route of administration, the concentration of test compound being administered, or whether the treatment uses a medicament that includes a protein, a nucleic acid encoding the test compound, or a cell capable of secreting a test compound as the active ingredient.

The pharmaceutical compositions of the present invention may be manufactured in a
25 manner that is itself known, *e.g.*, by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes. Proper formulation is dependent upon the route of administration chosen.

Dosing and Scheduling

Determination of an effective dose range for the medicaments of the present invention
30 is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. The therapeutically effective dose of a test compound can be estimated initially from cell culture assays and/or animal models. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the

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IC₅₀ (the dose where 50% of the cells show the desired effects) as determined in cell culture. Toxicity and therapeutic efficacy of test compounds also can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index (*i.e.*, the ratio between LD₅₀ and ED₅₀). Compounds which exhibit high therapeutic indices are preferable. The data obtained from these cell culture assays and animal studies may be used in formulating a dosage range for use in humans. The dosage of such compounds may lie within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. *See, e.g.*, Fingl *et al.*, (1975) in "The Pharmacological Basis of Therapeutics", Ch. 1 p1. Dosage amount and interval may be adjusted individually to provide plasma levels of the active test compound sufficient to maintain the desired effects.

Especially, for each of the aforementioned conditions, the compositions, *e.g.*, polypeptides and organic compounds, can be administered orally or via injection at a dose ranging from about 0.1 to about 250 mg/kg per day. The dose range for adult humans is generally from about 5 mg to about 17.5 g/day, preferably about 5 mg to about 10 g/day, and most preferably about 100 mg to about 3 g/day. Tablets or other unit dosage forms of presentation provided in discrete units may conveniently contain an amount which is effective at such dosage or as a multiple of the same, for instance, units containing about 5 mg to about 500 mg, usually from about 100 mg to about 500 mg.

The dose employed will depend upon a number of factors, including the age and sex of the subject, the precise disorder being treated, and its severity. Also the route of administration may vary depending upon the condition and its severity. In any event, appropriate and optimum dosages may be routinely calculated by those skilled in the art, taking into consideration the above-mentioned factors.

Generally, an efficacious or effective amount of one or more REG4 or KIAA0101 inhibitors is determined by first administering a low dose or small amount of a REG4 and/or KIAA0101 inhibitor and then incrementally increasing the administered dose or dosages, and/or adding a second REG4 and/or KIAA0101 inhibitor as needed, until a desired effect of inhibiting or preventing a cancer mediated by aberrant REG4 and/or KIAA0101

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overexpression or intracellular signalling is observed in the treated subject, with minimal or no toxic side effects. Applicable methods for determining an appropriate dose and dosing schedule for administration of a pharmaceutical composition of the present invention is described, for example, in Goodman and Gilman's The Pharmacological Basis of

5 Therapeutics, 11th Ed., Brunton, *et al.*, Eds., McGraw-Hill (2006), and in Remington: The Science and Practice of Pharmacy, 21st Ed., University of the Sciences in Philadelphia (USIP), Lippincott Williams & Wilkins (2005), both of which are hereby incorporated herein by reference.

Gene Therapy

10 The siRNA that inhibits expression of REG4 or KIAA0101, the antibody that neutralizes the activity of REG4, a cell-permeable dominant negative peptides identified as inhibits the interaction between KIAA0101/PCNA association may be therapeutically delivered using gene therapy to patients suffering from cancers. Alternatively, a polypeptide which comprises the neutralizing antibody or QKGIGEFF/SEQ ID NO: 46 of the present
15 invention can also be used as the peptides that directly alter the activity of REG4 or KIAA0101/PCNA association. In some aspects, gene therapy embodiments include a nucleic acid sequence encoding a suitable identified peptide of the invention. In preferred embodiments, the nucleic acid sequence includes regulatory elements necessary for expression of the peptide in a target cell. The nucleic acid may be equipped to stably insert
20 into the genome of the target cell (*see e.g.*, Thomas and Capecchi, (1987) *Cell* 51:503 for a description of homologous recombination cassettes vectors).

Delivery of the nucleic acids into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid-carrying vectors, or indirect, in which case, cells are first transformed with the nucleic acids *in vitro*, then transplanted into
25 the patient. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene therapy.

For general reviews of the methods of gene therapy, *see* Goldspiel *et al.*, (1993) *Clinical Pharmacy* 12:488-505; Wu and Wu, (1991) *Biotherapy* 3:87-95; Tolstoshev, (1993) *Ann. Rev. Pharmacol. Toxicol.* 33:573-96; Mulligan, (1993) *Science* 260:926-32; and Morgan
30 and Anderson, (1993) *Ann. Rev. Biochem.* 62:191-217; (1993) *Trends Biotechnol.* 11(5):155-215. Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel *et al.* (eds.), 1993, *Current Protocols in Molecular Biology*,

John Wiley & Sons, NY; and Kriegler, (1990), Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY.

Diagnosing the chemo-radiation therapeutic resistance of a cancer

The term “diagnosing” is intended to encompass predictions and likelihood analysis.

- 5 The present method is intended to be used clinically in making decisions concerning treatment modalities, including therapeutic intervention, diagnostic criteria such as disease stages, and disease monitoring and surveillance for cancer.

Method for diagnosing the chemo-radiation therapeutic resistance of a cancer

- 10 The expression of the REG4 was found to be specifically elevated in patients with the chemo-radiation therapy resistant pancreatic cancer in surgical sample. Therefore, REG4 gene identified herein as well as its transcription and translation products find diagnostic utility as a marker for the chemo-radiation therapeutic resistance of a cancer and by measuring the expression of REG4 gene in a cell sample, the chemo-radiation therapeutic resistance of a cancer can be diagnosed. Specifically, the present invention provides a
15 following method for diagnosing the chemo-radiation therapeutic resistance of a cancer by determining the expression level of the REG4 in the subject:

- [1] A method for diagnosing the chemo-radiation therapeutic resistance of a cancer in a subject, comprising a step of determining the expression level of REG4 gene in a subject-derived biological sample, and wherein an increase in the expression level as
20 compared to a normal control level of the gene indicates that the subject suffers from the cancer of the chemo-radiation therapeutic resistance or is at a risk of the chemo-radiation therapeutic resistance.

[2] The method of [1], wherein the expression level is at least 10% greater than the normal control level.

- 25 [3] The method of [1], wherein the expression level is determined by any of the methods selected from the group consisting of:

- (a) detecting mRNA of the gene;
- (b) detecting a protein encoded by the gene; and
- (c) detecting a biological activity of the protein encoded by the gene.

- 30 [4] The method of [3], wherein the expression level is determined by detecting hybridization of a probe to a gene transcript of the gene.

[5] The method of [4], wherein the hybridization step is carried out on a DNA array.

[6] The method of [3], wherein the expression level is determined by detecting the

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binding of an antibody against the protein encoded by REG4 gene as the expression level of the gene.

[7] The method of [1], wherein the biological sample comprises biopsy, sputum or blood.

5 [8] The method of [1], wherein the subject-derived biological sample comprises an epithelial cell.

[9] The method of [1], wherein the subject-derived biological sample comprises a cancer cell.

10 [10] The method of [1], wherein the subject-derived biological sample comprises a cancerous epithelial cell.

[11] The method of [1], wherein the cancer is pancreatic cancer.

A subject to be diagnosed by the present method is preferably a mammal. Exemplary mammals include, but are not limited to, e.g., human, non-human primate, mouse, rat, dog, cat, horse, and cow.

15 It is preferred to collect a biological sample from the subject to be diagnosed to perform the diagnosis. Any biological material can be used as the biological sample for the determination so long as it comprises the objective transcription or translation product of the REG4. The biological samples include, but are not limited to, bodily tissues and fluids, such as blood, sputum and urine. Preferably, the biological sample contains a cell population
20 comprising an epithelial cell, more preferably a cancerous epithelial cell or an epithelial cell derived from tissue suspected to be cancerous. Further, if necessary, the cell may be purified from the obtained bodily tissues and fluids, and then used as the biological sample.

According to the present invention, the expression level of the REG4 in the subject-derived biological sample is determined. The expression level can be determined at the
25 transcription (nucleic acid) product level, using methods known in the art. For example, the mRNA of the REG4 may be quantified using probes by hybridization methods (e.g., Northern hybridization). The detection may be carried out on a chip or an array. The use of an array is preferable for detecting the expression level of a plurality of genes (e.g., various cancer specific genes) including the REG4. Those skilled in the art can prepare such probes
30 utilizing the sequence information of the REG4 (SEQ ID NO: 1 GenBank Accession No.AY126670). For example, the cDNA of the REG4 may be used as the probes. If necessary, the probe may be labeled with a suitable label, such as dyes, fluorescent and isotopes, and the expression level of the gene may be detected as the intensity of the

hybridized labels.

Furthermore, the transcription product of the REG4 may be quantified using primers by amplification-based detection methods (e.g., RT-PCR). Such primers can also be prepared based on the available sequence information of the gene. For example, the primers (SEQ ID NO: 3 and 4) used in the Example may be employed for the detection by RT-PCR or Northern blot, but the present invention is not restricted thereto. Specifically, a probe or primer used for the present method hybridizes under stringent, moderately stringent, or low stringent conditions to the mRNA of the REG4. As used herein, the phrase “stringent (hybridization) conditions” is indicated in **Small interfering RNA** section.

Alternatively, the translation product may be detected for the diagnosis of the present invention. For example, the quantity of the REG4 protein may be determined. A method for determining the quantity of the protein as the translation product includes immunoassay methods that use an antibody specifically recognizing the protein. The antibody may be monoclonal or polyclonal. Furthermore, any fragment or modification (e.g., chimeric antibody, scFv, Fab, F(ab')₂, Fv, etc.) of the antibody may be used for the detection, so long as the fragment retains the binding ability to the REG4 protein. Methods to prepare these kinds of antibodies for the detection of proteins are well known in the art, and any method may be employed in the present invention to prepare such antibodies and equivalents thereof.

As another method to detect the expression level of the REG4 gene based on its translation product, the intensity of staining may be observed via immunohistochemical analysis using an antibody against the REG4 protein. Namely, the observation of strong staining indicates increased presence of the protein and at the same time high expression level of the REG4 gene. Furthermore, the translation product may be detected based on its biological activity.

The expression level of REG4 gene in a biological sample can be considered to be increased if it increases from the control level of REG4 gene by, for example, 10%, 25%, or 50%; or increases to more than 1.1 fold, more than 1.5 fold, more than 2.0 fold, more than 5.0 fold, more than 10.0 fold, or more.

The control level may be determined at the same time with the test biological sample by using a sample(s) previously collected and stored from a subject/subjects whose disease state (cancerous or non-cancerous) is/are known. Alternatively, the control level may be determined by a statistical method based on the results obtained by analyzing previously determined expression level(s) of the REG4 gene in samples from subjects whose disease

state are known. Furthermore, the control level can be a database of expression patterns from previously tested cells. Moreover, according to an aspect of the present invention, the expression level of the REG4 gene in a biological sample may be compared to multiple control levels, which control levels are determined from multiple reference samples. It is preferred to use a control level determined from a reference sample derived from a tissue type similar to that of the patient-derived biological sample. Moreover, it is preferred, to use the standard value of the expression levels of the REG4 gene in a population with a known disease state. The standard value may be obtained by any method known in the art. For example, a range of mean $\pm$ 2 S.D. or mean $\pm$ 3 S.D. may be used as standard value.

In the context of the present invention, a control level determined from a biological sample that is known not to be cancerous is called "normal control level". On the other hand, if the control level is determined from chemo-radiation therapeutic resistant biological sample, it will be called "chemo-radiation therapeutic resistance control level".

When the expression level of the REG4 gene is increased compared to the normal control level or is similar to the chemo-radiation therapeutic resistance control level, the subject may be diagnosed to be suffering from the cancer with the chemo-radiation therapeutic resistance or at a risk of the chemo-radiation therapeutic resistance.

Difference between the expression levels of a test biological sample and the control level can be normalized to the expression level of control nucleic acids, e.g., housekeeping genes, whose expression levels are known not to differ depending on the cancerous or non-cancerous state of the cell. Exemplary control genes include, but are not limited to, β -actin, glyceraldehyde 3 phosphate dehydrogenase, and ribosomal protein P1.

Aspects of the present invention are described in the following examples, which are not intended to limit the scope of the invention described in the claims.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

Example 1:

1. General Methods

Clinical samples

Pre-operative and post-operative (3 or 4 weeks after the curative resection) serum samples were obtained with informed consent from seven patients who underwent curative resection for pancreatic ductal adenocarcinoma. Conventional paraffin-embedded tissue sections of PDACs were also obtained from surgical specimens that had been resected at the same center. Tissue microarray samples, where 31 PDAC tissues and 2 endocrine-tumor tissues were spotted in duplicate, were obtained from ISU ABXIS (Seoul, Korea).

Cell lines

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 (ATCC, Rockville, MD). These cell lines were grown in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) for PK-45P and SUIT-2, Dulbecco's Modified Eagle's Medium (Sigma-Aldrich) for MIAPaCa-2 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% CO₂. FreeStyle™ 293 cells (Invitrogen, Carlsbad, CA) were suspended in FreeStyle™ 293 Expression Medium (Invitrogen) and were grown in Erlenmeyer flask (Corning, NY) rotated on an orbital shaker platform at 125 rpm. Cells were maintained at 37°C in an atmosphere of humidified air with 8% CO₂. Pancreatic cancer cell lines MIA-PaCa2 and Panc-1, and normal rodent cell line NIH3T3 were purchased from the American Type Culture Collection (ATCC, Rockville, MD), which were grown in Delbecco's modified Eagle's medium or RPMI1640 (Sigma-Aldrich, St. Louis, MO). Pancreatic cancer cell lines PK-59, KLM-1, PK-45P, and PK-1 were provided by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan) and maintained in RPMI1640 (Sigma-Aldrich); both media were supplemented with 10% fetal bovine serum (Cansera International, Ontario, Canada) and 1% antibiotic/antimycotic solution (Sigma-Aldrich). Cells were maintained at 37°C in atmospheres of humidified air with 5% CO₂.

2. Semi-quantitative RT-PCR for REG4

Purification of PDAC cells, pancreatic cancer cells and normal pancreatic ductal epithelial cells were described previously (Nakamura T, *et al.* Oncogene 2004; 23: 2385-400.); RNAs from the purified cell populations and from normal human heart, lung, liver, kidney, brain, pancreas (BD Biosciences, Palo Alto, CA) and normal pancreatic ductal cells

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were subjected to two rounds of amplification by T7-based *in vitro* transcription (Epicentre Technologies, Madison, WI) and subsequent synthesis of single-strand cDNA. Total RNAs from human pancreatic cancer cell lines were extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendation. Extracted RNAs were
 5 treated with DNase I (Roche, Mannheim, Germany) and reverse-transcribed to single-stranded cDNAs using oligo (dT) primer with Superscript II reverse transcriptase (Invitrogen). The present inventors prepared appropriate dilutions of each single-stranded cDNA for subsequent PCR amplification by monitoring α -tubulin (TUBA) as a quantitative control. The primer sequences were

10 5'-AAGGATTATGAGGAGGTTGGTGT-3' (SEQ ID NO: 9) and
 5'-CTTGGGTCTGTAACAAAGCATTC-3' (SEQ ID NO: 10) for *TUBA*;
 5'-CCAATTGCTATGGTTACTTCAGG-3' (SEQ ID NO: 3) and
 5'-GAAAAACAAGCAGGAGTTGAGTG-3' (SEQ ID NO: 4) for *REG4*.
 5'-AGCTTTGTTGAACAGGCATTT-3' (SEQ ID No.;26) and
 15 5'-GGCAGCAGTACAACAATCTAAGC-3' (SEQ ID No.;27) for KIAA0101
 (NM_014736, amino acid sequence set forth in SEQ ID No.;40 encoded by nucleotide
 sequence set forth in SEQ ID No.;39),

5'-CACCCCCACTGAAAAAGAGA-3' (SEQ ID No.;28) and
 5'-TACCTGTGGAGCAAGGTGC-3' (SEQ ID No.;29) for *β 2MG*.

20 All reactions involved initial denaturation at 94°C for 2 minutes followed by 23
 cycles (for *TUBA*) or 28 cycles (for *REG4*) at 94°C for 30 seconds, 58°C for 30 seconds, and
 72°C for 1 minute or 95°C, 5-min initial denaturation step followed by 23 cycles (*β 2MG*) or
 28 cycles (for KIAA0101) at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, on a GeneAmp
 PCR system 9700 (PE Applied Biosystems, Foster, CA).

25 3. Antibody for REG4

The expressing vector of His-tagged full-length human REG4 was transfected into
 293T cells and recombinant REG4 (REG4-His) was purified from its culture media by the use
 of TALON Purification Kit (Clontech, San Diego, CA). The REG4-His protein was
 prepared for injection by emulsifying the antigen solution with Freund's complete adjuvant.
 30 Polyclonal anti-REG4 antibody (pAb) was raised in rabbits (Medical & Biological
 Laboratories, Nagoya, Japan) against the REG4-His protein, and the immune sera were
 purified on affinity columns according to standard protocol. Mouse monoclonal antibody

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(mAb) was also raised by inoculating REG4-His into BALB/C mice with Freund's complete adjuvant. Their lymphocytes were fused with myeloma cell P3U1 and monoclonal hybrid cells were generated and validated by the standard technique.

4. Antibodies and recombinant protein for KIAA0101.

5 The cDNA fragment encoding full-length KIAA0101 (111 amino-acids, NP_055551) (SEQ ID No.;15) was generated using KOD-Plus polymerase (TOYOBO) and cloned into pET21 vector (Novagen). The recombinant KIAA0101 protein fused with polyhistidine tag at COOH terminus was expressed in *E. coli*, BL21 codon plus (Stratagene), and purified with Ni-NTA resin (QIAGEN) under native condition according to the supplier's protocol.

10 Further purification was performed by use of High Performance Liquid Chromatography AKTA explorer (Amersham) equipped with MonoS HR 5/5 (Amersham). The protein was immunized into rabbits, and the immune sera were purified on affinity-columns packed with Affi-Gel 10 activated affinity media (Bio-Rad) conjugating recombinant KIAA0101 protein with accordance of basic methodology. The affinity-purified anti-KIAA0101 polyclonal
15 antibody was used for detection of KIAA0101 protein.

5. Immunohistochemical staining for REG4.

The sections were deparaffinized and autoclaved for 15 minutes at 108°C in citrate buffer, pH 6.0. Endogenous peroxidase activity was quenched by incubation for 30 minutes in 0.33% hydrogen peroxide diluted in methanol. After incubation with fetal bovine serum
20 for blocking, the sections were incubated with anti-REG4 pAb for 1 hour at room temperature (1:1000). After washing with PBS, immunodetection was performed with peroxidase-labeled anti-mouse immunoglobulin (Envision kit, Dako Cytomation, Carpinteria, CA). Finally, the reactants were developed with 3, 3'-diaminobenzidine (Dako) and the cells were counter-stained with hematoxylin.

25 Through genome-wide cDNA microarray analysis, the present inventors identified dozens of genes that were over-expressed in PDAC cells (Hartupée JC, *et al.* Biochim Biophys Acta 2001; 1518: 287-93.). Among them, the present inventors focused on REG4 for which the present inventors confirmed its over-expression by RT-PCR in seven of the nine microdissected PDAC cell populations examined (Fig. 1A). Its mRNA levels in PDAC cells
30 were apparently higher than that of normal pancreas and vital organs including heart, lung, kidney, and brain.

Immunohistochemical analysis using pAb to REG4 at another series of PDAC

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tissues revealed strong signals of REG4 Goblet cell-like vesicles (Fig.1B) or at the cell surface (Fig.1C) of cancer cells, while acinar cells in normal pancreas showed faint staining of 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 also (data not shown). In addition, tissue-microarray with other series of 31 PDAC tissues spotted showed that 14 of 31 PDACs expressed high levels of REG4, and totally 35 out of 64 PDACs (55%) showed positive staining by anti-REG4 antibody. Well-differentiated PDACs (G1) showed positive staining for REG4 more frequently than less differentiated (G2, G3, and G4) PDACs ($p=0.0001$ by χ^2 test).

6. Establishment of stably REG4-expressing cells.

To create REG4 expression vector, the entire coding sequence of REG4 cDNA was amplified by PCR using the primer pair with restriction enzyme sites;

5'-CGGAATTCATGGCTTCCAGAAGCATGC-3' (SEQ ID NO.;63) and

5'-ATAAGAATGCGGCCGCTGGTTCGGTACTTGCACAGG-3' (SEQ ID

NO.;64)

which contained EcoRI and NotI restriction sites indicated by the first and second underlines, respectively. The product was inserted into the EcoRI and NotI sites of pCAGGSnHC for expressing a HA-tagged protein. The plasmid was transfected into REG4-negative PDAC cell line, PK-45P cells, using FuGENE6 (Roche) according to the manufacture's recommended procedures. A population of cells was selected with 0.5 mg/ml Geneticin (Invitrogen), and clonal PK-45P cells were sub-cloned by limiting dilution. To assess the levels of HA-tagged REG4 expression, several clones were harvested in a lysis buffer containing 50mM Tris-HCl, pH 7.4, 150mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1mM EDTA, 0.1% protease inhibitor cocktail III (Calbiochem, San Diego, CA). Samples were centrifuged and the pellet was discarded. The amount of protein present in the supernatant was measured by Bradford method. Aliquots of 10 μ g were subjected to 15% SDS-PAGE and detected by western blotting using anti-HA antibody (3F10) (Roche). The amount of each protein was normalized by and anti- β -actin antibody (Sigma-Aldrich). Three clones that expressed REG4 constitutively were established (C1-6, C2-6, C10). Control PK-45P cells transfected with an empty pCAGGSnHC-HA vector was also established (M1, M3, M6).

7. γ -ray and gemcitabine resistance assay.

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3,000 cells of each of REG4-expressing clones (C1-6, C2-6, C10) or control clones (M1, M3, M6) was seeded into each well of a 96-well culture dish and incubated in the medium containing 10% FBS. After 48 hours pre-incubation, cells were γ -irradiated at 1, 5, 10, or 30 Gy using a ^{60}Co source, or treated with 0.1-100,000 nM gemcitabine for 48 hours. After 48 hours, viable cells were measured by using Cell-counting kit-8 (DOJINDO). To evaluate % inhibition value, relative ratio of absorbance-(each treatment)/absorbance-(no treatment control) was calculated. For FACS analysis, 48,000 cells/well were seeded in 6-well plate, and after 48 hours pre-incubation, cells were γ -irradiated at 0, 1, or 5 Gy using a ^{60}Co source, or treated with 10nM or 50nM gemcitabine for 48 hours. After 48 hours, cells were collected, washed with PBS, fixed with cold 70% ethanol, stained with propidium iodide (10 $\mu\text{g}/\text{ml}$) and ribonuclease A (100 $\mu\text{g}/\text{ml}$), and subjected to cell cycle analysis using FACSCalibur™ Flow Cytometry System (BD) analysis. The percentage of aneuploid cells was calculated with cell cycle analysis software (BD CELLQuest™).

8. Immunohistochemical staining for KIAA0101.

Conventional sections from pancreatic cancer tissues were obtained from surgical specimens that were resected under the appropriate informed consent. Sections from normal pancreas were purchased from Biochain (Hayward, CA). The sections were deparaffinized and autoclaved at 108°C in Dako Cytomation Target Retrieval Solution High pH (Dako, Carpinteria, CA) for 15 min. After blocking of endogenous peroxidase and proteins, the sections were incubated with anti-KIAA0101 antibody (diluted by 1:200) at room temperature for 30 min. After washing with PBS, immunodetection was performed with peroxidase labeled anti-rabbit immunoglobulin (Envision kit, Dako). Finally, the reactants were developed with 3, 3'-diaminobenzidine (Dako). Counterstaining was performed using hematoxylin.

9. Northern blot analysis.

The present inventors extracted total RNAs from several pancreatic cancer cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA) and performed Northern blot analysis. After treatment with DNase I (Nippon Gene, Osaka, Japan), mRNA was purified with Micro-FastTrack (Invitrogen), according to the manufacturer's protocols. A 1- μg aliquot of each mRNA from pancreatic cancer cell lines, as well as those isolated from normal human adult heart, lung, liver, kidney, brain, and pancreas (BD Biosciences, Palo Alto, CA), were separated on 1% denaturing agarose gels and transferred onto nylon membranes. The 702-

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bp probe specific to KIAA0101 was prepared by PCR using the following primer set: forward 5'-AGCTTTGTTGAACAGGCATTT-3' (SEQ ID No.;1) and reverse

5'-GGCAGCAGTACAACAATCTAAGC-3' (SEQ ID No.;2). Hybridization with a random-primed, $\alpha^{32}\text{P}$ -dCTP-labeled probe was carried out according to the instructions for

5 Megaprime DNA labeling system (Amersham Biosciences, Buckinghamshire, UK).

Prehybridization, hybridization and washing were performed according to the supplier's recommendations. The blots were auto-radiographed with intensifying screens at -80°C for 10 days.

10. Small interfering RNA (siRNA)-expressing constructs and transfection for knock down KIAA0101.

To knock down endogenous KIAA0101 expression in pancreatic cancer cells, the present inventors used psiU6BX3.0 vector for expression of short hairpin RNA against a target gene as described previously (Taniuchi et al., (2005) Cancer Res, 65: 105-12). The target sequences of the synthetic oligonucleotides for siRNA for KIAA0101 were as follows:,
15 #759si; 5'-GCCATATTGTCACTCCTTCTA-3' (SEQ ID No.; 7), and EGFPsi; 5'-GAAGCAGCAGCACTTCTTC-3' (SEQ ID No.; 8) (as a negative control). Pancreatic cancer cell lines KLM-1 and MIA-PaCa2, which highly expressed KIAA0101, were plated onto 10 cm plates, and transfected with 8 μg plasmid designed to express siRNA to KIAA0101 using FuGENE6 (Roche) according to manufacture's instruction.

20 Cells were selected by 0.5 mg/ml (for KLM-1) or 0.8 mg/ml (for MIA-PaCa2) of Geneticin (Sigma-Aldrich) for 5 days, and then harvested to analyze knockdown effect on KIAA0101 expression. For colony formation assay, transfectants expressing siRNAs were grown for 14 days in media containing Geneticin. After fixation with 4% paraformaldehyde, transfected cells were stained with Giemsa solution to assess colony formation. Cell
25 viability was quantified using Cell counting kit-8 (DOJINDO, Kumamoto, Japan). After 14 days of culture in the Geneticin-containing medium, the solution was added at a final concentration of 10%. Following incubation at 37°C for 3 hours, absorbance at 450nm was measured with a Microplate Reader 550 (Bio-Rad, Hercules, CA).

11. Establishment of exogenous KIAA0101-expressing cells and their growth in vitro and in vivo.

30 KIAA0101 cDNA was prepared by PCR amplification using the forward primer that included the Kozak sequence and NotI linker, and the reverse primer including a NotI

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linker. The PCR product was inserted into the NotI sites of the mammalian expression vector, pCAGGS/HA for expressing a HA-tagged protein. The pCAGGS-KIAA0101-HA or empty pCAGGS/HA mock vector was transfected into PK-45P, which showed faint expression of KIAA0101, and NIH3T3 cells, which exhibited hardly detectable expression of mouse KIAA0101 homologue (NP_080791), by FuGENE6 (Roche) according to the manufacturer's protocol.

Then, the Geneticin-resistant clones were selected in the culture medium containing 0.5 mg/ml for PK-45P and 0.9 mg/ml for NIH3T3 of Geneticin. The exogenous KIAA0101 expression in each clone was confirmed by Western-blot analysis using anti-FLAG tag antibody (Sigma-Aldrich) and anti- β -actin antibody (Sigma-Aldrich). For growth assay, 7,500 cells of each of KIAA0101 expressing clone (PK45P-KIAA0101) or control clone (PK45P-Mock) was seeded into each well of a 24-well culture dish and incubated in the medium containing 10% FBS. Cell viability was quantified with MTT assay. The experiment was repeated at least three times. For in vivo transformation, 5×10^6 cells of one stable clone NIH3T3-KIAA0101 and one NIH3T3-Mock were inoculated in the right and left flank of 8-week nude mice, respectively, and the tumors were harvested after four weeks. Each of the tumors was weighted and immunostained by anti-KIAA0101 antibody.

12. Immunoprecipitation and mass-spectrometric analysis for KIAA0101-associated complexes.

To isolate proteins that associated with KIAA0101 protein, the present inventors performed immunoprecipitation experiments using the anti-KIAA0101 antibody. Pancreatic cancer cell lines KLM-1 and PK59, which over-expressed KIAA0101, were lysed in lysis buffer (50mM Tris-HCl pH8.0, 150mM NaCl, 0.5% NP-40, Protease Inhibitor Cocktail Set III [Calbiochem, San Diego, CA]). Equal amounts of total proteins were incubated at 40°C for 1h with 2 μ g of anti-KIAA0101 polyclonal antibody or a rabbit IgG (Santa Cruz Biotechnologies, Santa Cruz, CA). Immunocomplexes were incubated with protein G Sepharose (Zymed Laboratories, South San Francisco, CA) for 1h and washed with lysis buffer.

Co-precipitated proteins were separated in 5-20% gradient SDS-PAGE and stained by silver-staining kits (Wako, Osaka, Japan). Bands that differentiated proteins precipitated with anti-KIAA0101 polyclonal antibody from those precipitated with control IgG were excised, digested in-gel with trypsin, and analyzed for peptide-mass fingerprints using an

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AXIMA-CFR MALDI-TOF mass spectrometer (Shimadzu Corporation, Tsukuba, Japan). Peptide masses were searched with 10-ppm mass accuracy, and protein-database searches were performed using the database-fitting program IntelliMarque (Shimadzu). The protein binding identified by this strategy was validated by immunoprecipitation using anti-
 5 KIAA0101, anti-PCNA antibody (Santa-Cruz), anti-POLD1 antibody (Santa-Cruz) and anti-FEN1 antibody (Santa-Cruz).

13. Cell-permeable peptide treatment and KIAA0101-PCNA interaction.

To inhibit the interaction between KIAA0101 and PCNA in the dominant-negative manner, the present inventors designed the PIP box motif peptide of KIAA0101 conjugating
 10 with arginine (R)-repeat cell-permeable peptide (Noguchi et al., (2004) Nat Med., 10: 305-9). PIP20 was RRRRRRRRRRRRGGG-VRPTPKW{qKGiGEff}RLSPK (SEQ ID No.; 9) (PIP box motif is shown in parentheses, and the conserved residues are shown as lower case). PIP20mut replaced the conserved residues in the PIP box motif with alanine:
 RRRRRRRRRRRRGGG-VRPTPKW{aKGaGEaa}RLSPK (SEQ ID No.; 10). Scramble
 15 peptide was also designed as a negative control; RRRRRRRRRRRRGGG-IFKQWPRGETKPRVLSKGF (SEQ ID No.; 11). Furthermore, the present inventors also designed shorter peptide containing the PIP box motif, PIP16: RRRRRRRRRRRRGGG-TPKW{qKGiGEff}RLSP (SEQ ID No.; 12), PIP16mut; RRRRRRRRRRRRGGG-TPKW{aKGaGEaa}RLSP (SEQ ID No.; 13). They were synthesized by Sigma-Aldrich and
 20 purified by HPLC to more than 95% grade. Cancer cell line KLM-1 with KIAA0101 expression and normal mouse cell line NIH3T3 without expression of mouse KIAA0101 homologue were treated with serial concentration (5μM, 10μM, and 20μM) of each of these cell-permeable peptides. At day1 and day3, the cells were exposed with each peptide, and at day5 viable cell number was evaluated by MTT assay, described above.

25 Example 2

1. siRNA-expressing vector and colony formation assay/MTT assay.

To knock down endogenous REG4 expression in PDAC cells, the present inventors used psiU6BX3.0 vector for expression of short hairpin RNA against a target gene as described previously (Shimokawa T, *et al.* Cancer Res 2003; 63: 6116-20.). The target
 30 sequences of the synthetic oligonucleotides for siRNA for REG4 were as follows: REG4-si2; 5'-GACAGAAGGAAGAACTCA-3' (SEQ ID NO: 5), and EGFPsi; 5'-GAAGCAGCACGACTTCTTC-3' (SEQ ID NO: 6) (as a negative control). PDACs cell

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lines, SUIT-2 (REG4-positive) and MIAPaCa-2 (REG4-negative), were plated onto 6-well plates, and transfected with plasmid designed to express siRNA (10 μ g) using FuGENE6 (Roche, Basel, Switzerland) according to manufacture's instruction. Plasmids expressing siRNAs were prepared by cloning double-stranded oligonucleotides. The sequences of

5' paired oligonucleotides are;
5'-CACCGACAGAAGGAAGAACTCATTCAAGAGATGAGTTTCTTCCTTCTGTC-3'
(SEQ ID NO; 11) and

5'-AAAACTGTCTTCCTTCTTTGAGTAAGTTCTCTACTCAAAGAAGGAAGACAG-3'
(SEQ ID NO; 12) for si2;

Cells were selected by 0.9 mg/ml (for SUIT-2) or 0.8 mg/ml (for MIAPaCa-2) of Geneticin (Sigma-Aldrich) for 7 days, and then harvested to analyze knockdown effect on REG4 expression. For colony formation assay, transfectants expressing siRNAs 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 hours, absorbance was measured at 490 nm and at 630 nm as a reference with Microplate Reader 550 (Bio-Rad, Hercules, CA).

To examine roles of REG4 over-expression in PDAC cell growth, the present inventors constructed several expression vectors designed to express siRNA specifically to REG4 and transfected them into PDAC cell line SUIT-2, which expressed REG4 endogenously at high level. Among the three plasmids the present inventors tested in SUIT-2 cells, REG4-si2 showed the significant knockdown effect on endogenous REG4 transcript (Fig.2A), and this transfection resulted in reduction of the viable cells measured by MTT assay (Fig.2B) as well as those of the numbers of colonies (Fig.2C), whereas the transfection of other plasmids (a negative control of siEGFP) showed no knockdown effect on REG4 expression and the cell growth of SUIT-2. On the other hand, REG4-si2 did not affect the cell viability of MIAPaCa-2, which did not express REG4, excluding a possibility of the "off-targeting" effect of REG4-si2 (data not shown). The growth suppressive effect of this siRNA-expressing vector (REG4-si2) was correlated well with their gene-silencing effects, and these data indicated a critical role of REG4 in pancreatic cancer cell survival and/or growth.

Example 3

1. Generating bio-active recombinant human REG4.

To create the bio-active form of REG4, the entire coding sequence of REG4 cDNA was amplified by PCR using the primer pair with restriction enzyme sites;

- 5 5'-CG{GAATTC}ATGGCTTCCAGAAGCATGC-3' (forward) (SEQ ID NO: 7) and
5'-ATAAGAAT{GCGGCCGC}TGGTCGGTACTTGACACAGG-3' (reverse) (SEQ ID NO: 8) which contained *EcoRI* and *NotI* restriction sites shown in parentheses, respectively. The product was inserted into the *EcoRI* and *NotI* sites of pCAGGS for expressing a HA-tagged protein. FreeStyle™ 293 cells were seeded at 1.5×10^5 cells/ml in 30 ml medium. REG4-
10 HA/pCAGGS vectors were transfected with cells using FuGene 6, according to the instruction manuals. Culture medium was harvested after 48 hours and recombinant human REG4 (rhREG4) was purified with HA agarose (Sigma-Aldrich).

2. Immunoprecipitation.

- SUIT-2 cells cultured in 10-cm dish were washed and further cultured for 2 days in
15 serum-free medium. After centrifugation at 10,000xg and 4°C for 15 minutes, the supernatant was treated protein G Sepharose (Zymed Laboratories, San Francisco, CA) for 1 hour at 4°C. Then pretreated supernatant was added to a mixture of protein G Sepharose, which was pre-incubated with anti-REG4 mAb. Incubation was carried out with gentle rotation at 4 °C for 4 hours followed by two washing steps. Bound proteins were eluted and
20 separated on a 15% SDS-PAGE. After electrophoresis separation, proteins were transferred to nitrocellulose membranes (Amersham) and probed with anti-REG4 pAb. Protein bands were visualized by chemiluminescent detection system (ECL, Amersham).

3. Akt Phosphorylation.

- To assess the levels of phosphorylated Akt, PK-45P cells were treated with 0, 0.1, 1
25 or 10nM rhREG4 and with or without 100 µg/ml anti-REG4 mAb for 6 hours. Following treatment, the cells were washed with cold PBS and harvested in a lysis buffer containing 50 mM HEPES, pH 7.5, 200 mM NaCl, 2.5 mM EDTA, 2.5mM EGTA, 10mM NaF, 1mM Na₃VO₄, 0.5% Triton X-100, 0.5 mM 1,4-dithiothreitol, 0.1% protease inhibitor cocktail III (Calbiochem, San Diego, CA). Samples were centrifuged and the pellet was discarded.
30 The amount of protein present in the supernatant was measured by Bradford method. Aliquots of 20µg were subjected to 10% SDS-PAGE and detected by western blotting using

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anti-pSer473 Akt antibody (Abcam, Cambridge, MA). The total amount of Akt protein was evaluated by anti-Akt antibody (Santa Cruz Biotechnology, Inc., CA).

To examine an effect of secreted REG4 on pancreatic cancer cell growth, the present inventors generated bio-active rhREG4 protein by using mammalian system (FreeStyleTM 293-F) (Fig.3A), and performed cell growth assay by treating PK-45P cells, which showed low expression of REG4, with several concentration (0-10nM) of rhREG4. Fig.4B showed that the presence of REG4 protein in culture medium clearly stimulated cell proliferation dose-dependently, which implicated that secreted REG4 functions to promote cell proliferation extracellularly and in autocrine/paracrine manner. One of the downstream targets of REG family was reported to be Akt signaling pathway (Sekikawa A, *et al.* Gastroenterology 2005; 128: 642-53., Bishnupuri KS, *et al.* Gastroenterology 2006; 130: 137-49.). To examine whether our rhREG4 can activate Akt signaling pathway in PDAC cells, PK-45P cells were incubated at the presence of serial doses of rhREG4 and the phosphorylated Akt was detected by Western blot analysis using the antibody specific to Akt with 473 serine phosphorylated. The rhREG4 treatment clearly resulted in increasing phosphorylation of Akt (Fig.3C), while the total expression level of Akt was not changed by the treatment of rhREG4. These data indicated REG4 stimulated cell growth through Akt signaling pathway in PDAC cells.

To evaluate the therapeutic potential of anti-REG4 mAb, the present inventors performed cell growth assay by treating PDAC cells with anti-REG4 mAb. At first, the present inventors checked binding affinity of several anti-REG4 mAbs by immunoprecipitation using cell culture medium. Fig.4A showed that one anti-REG4 mAb (34-1) binds 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, while the control antibody did not show any neutralizing activity (Fig.4B). And the growth assay using SUIT-2 cells expressing endogenous REG4 at high level showed that anti-REG4 mAb treatment inhibited SUIT-2 cell growth dose-dependently (Fig.4C), while anti-REG4 mAb did not affect the cell growth of MIAPaCa-2 that did not express REG4 at all.

Furthermore, the present inventors also examined the effect on Akt phosphorylation by treating PK45P cell with rhREG4 and anti-REG4 mAb, and anti-REG4 mAb treatment suppressed Akt phosphorylation in PDAC cells which was induced by the treatment of rhREG4 (Fig.4D), indicating that anti-REG4 mAb was likely to inhibit Akt signaling

pathways in PDAC cells by neutralizing secreted REG4 and shutting down its autocrine/paracrine pathways. Taken together, these data suggested that anti-REG4 antibody has neutralization activity to cell proliferation stimulated by REG4.

To evaluate the therapeutic potential of anti-REG4 mAb, the present inventors performed tumor growth assay by treating PDAC cells inoculated mice with anti-REG4 mAb. 5 5×10^6 cells of REG4-expressing PDAC cell line SUIT2 were inoculated in the flank of 8-week nude mice, and the long (L) and short diameters (S) of the tumors were measured twice a week. The tumor volumes were calculated by $L \times L \times S \times 0.52$. The antibody treatment with anti-REG4 mouse mAb 34-1 (300 μ g/mouse i.p.) started when the tumor volume reached 10 100-200 mm³. The antibody was treated two times per a week intraperitoneally, and as a control, non-specific mouse IgG (Acris) was also treated at the same schedule. As shown in Figure 5A, 34-1 mAb treatment suppressed the tumor growth ($P=0.0598$) and at day 30, the tumors were harvested and weighted. 34-1 mAb treatment suppressed the tumor weights significantly (Fig. 5B, $P=0.0489$).

15 Example 4

1. REG4 expression in chemo-radiation therapy resistant PDAC in vitro.

To investigate how REG4 expression can contribute to the resistance of PDAC to chemo-radiation therapy, we generated three clones that constitutively expressed REG4 from REG4-negative PDAC cells and treated these clones with γ -radiation or gemcitabine in vitro.

20 Western blot analysis confirmed exogenous REG4 expression in C1-6, C2-6, and C10 clones, but not Mock clones (Fig. 6A). Comparing with non-irradiation, γ -radiation suppressed cell viability of REG4-expressing cells and Mock cells. However, as shown in Fig. 6B, REG4-expressing cells were less sensitive to γ -radiation, and after 30-Gy γ -radiation, viability of REG4-expressing cells (C1-6, C10, C2-6) were suppressed to 60% comparing with non-irradiation, while Mock cell viability were suppressed to less than 40% ($P<0.001$). FACS 25 analysis after γ -radiation demonstrated that 1-Gly γ -radiation induced 28.7% of sub-G1 population (apoptotic cells) of Mock cells, while only 10.73% of REG4-expressing cells (Fig. 6C). Furthermore, 5-Gly γ -radiation induced 46.47 % of sub-G1 population (apoptotic cells) of Mock cells, while only 24.68 % of REG4-expressing cells (Fig. 6C). These finding 30 implicated that REG4 expression in PK-45P cells could strongly contribute to the resistance to γ -radiation.

Next, REG4-positive or negative cells were treated with serial concentration of

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gemcitabine, and evaluated their viability. As shown in Fig. 7A, REG4-expressing cells (C1-6, C10, C2-6) were less sensitive to gemcitabine, comparing with Mock cells. IC₅₀ of gemcitabine to REG4-expressing cells was about 100nM at C1-6 and C10, and about 50nM at C2-6, which showed less expression of REG4 than C1-6 and C10 (Fig. 6A), while IC₅₀ of Mock cells was about 30nM. But this difference is not so dominant. Furthermore, FACS analysis showed some trend that REG4-expressing cells showed less apoptotic cells after 50nM gemcitabine treatment, but it is not statistically significant (Fig. 7B). Treatment of higher concentration of gemcitabine did not reveal significant difference of apoptotic cells. These findings showed REG4 expression in PK-45P cells might have some contribution to the resistance to gemcitabine, but this contribution is not so strong. Hence, REG4 expression in PDAC cells is likely to contribute to its resistance to radiation, rather than gemcitabine-based chemotherapy.

2. REG4 expression in the neo-adjuvant chemo-radiation therapy resistant patient.

In order to evaluate the association between REG4 expression and therapeutic resistance of pancreatic cancers, the surgical specimens of the patients, who underwent the neo-adjuvant chemo-radiation therapy (neo-CRT) followed by surgical resection of their pancreatic adenocarcinomas, were investigated. They were treated with 3D confocal radiation therapy (total 50 Gy) and, concurrently, gemcitabine (1g/m²/week for 3 weeks), followed by 8-week follow-up treatment. 19 surgical specimens were immunostained with anti-REG4 antibody and REG4 expression and the pathological response to neo-CRT were evaluated. Among 19 neo-CRT specimens, 9 samples showed histological response to neo-CRT, while 10 did not. Regarding to REG4 expression, only 2 out of 9 responders showed REG4 expression in the tumor cells (Fig. 8A and B), while 7 out of 10 non-responders showed REG4 expression (Fig. 8C and D). This REG4 expression was significant associated with the response to neo-CRT (chi² test, P=0.0028), implicating that REG4 expression could make cancer cells survived under the cytotoxic treatments such as chemotherapy and radiation therapy.

Example 5

Over-expression of *KIAA0101* in pancreatic cancer cells.

Among a number of genes that were over-expressed in pancreatic tumor cells on a genome-wide cDNA microarray analysis (Nakamura T., *Oncogene*, 23: 2385-400, 2004.), the present inventors focused on KIAA0101 for this study. The present inventor's microarray data had shown over-expression of KIAA0101 in all of the informative cases of pancreatic

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cancer cells the present inventors examined (14 out of 14 informative cases showed more than 10 folds of expression signal), and its over-expression was confirmed by RT-PCR in eight of the nine microdissected pancreatic cancer cell populations examined (Fig. 9A). Northern-blot analysis using a KIAA0101 cDNA fragment as the probe identified a transcript of about 1.2 kb that was highly expressed in all cancer cell lines the present inventors examined; no expression was observed in any vital organs including lung, heart, liver and kidney (Fig. 9B). Immunohistochemical analysis using a polyclonal antibody to KIAA0101 also showed strong signals in the nuclei of pancreatic cancer cells in all of the pancreatic cancer tissue sections from five additional patients (Fig. 9C). No staining was observed in normal pancreatic epithelia and acinar cells (Fig. 9C) and in the vital normal organs including lung, heart, liver and kidney.

Example 6

Effect of *KIAA0101* knockdown by siRNAs on growth of pancreatic cancer cells.

To investigate for the biological significance of KIAA0101 overexpression in cancer cells and its potentials as a molecular target for cancer therapy, the present inventors constructed several siRNA-expression vectors specific to KIAA0101 mRNA sequences and transfected them into KLM-1 and MIA-PaCa2 that endogenously express high levels of KIAA0101 mRNA. A knockdown effect was confirmed by RT-PCR when the present inventors used #759si constructs (Fig. 10A). Colony-formation assays (Fig. 10B) and MTT assays (Fig. 10C) using KLM-1 revealed a drastic reduction in the number of cells transfected with #759si, compared with EGFPsi for which no knockdown effect was apparent. Similar effects were obtained with the MIA-PaCa2 cell line. These findings indicated that KIAA0101 is likely to play some important roles of cancer cell proliferation and inhibition of KIAA0101 function can have some potential as a novel molecular target for cancer therapy.

Example 7

Exogenous overexpression of KIAA0101 promoted cancer cell growth and transformed NIH3T3.

To further explore the potential oncogenic property of KIAA0101, the present inventors established several clones of PK45P-derivative cell lines, PK45P-KIAA0101, in which exogenous KIAA0101 expressed constitutively. The present inventors also prepared control PK-45P cells transfected with the mock vector (PK45P-Mock) and compared their growth rates. Western blot analysis (Fig. 11A) validated exogenous KIAA0101 expression

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in six clones. The growth curve measured by MTT assay demonstrated that the six clones of PK45P-KIAA0101 (solid lines) grew significantly more rapidly than the four PK45P-mock clones (dash lines, Fig. 11B), indicating the KIAA0101 expression enhanced proliferation of cancer cells.

5 Among the cell line the present inventors examined, only normal mouse NIH3T3 cell line did not showed any expression of KIAA0101 homologue and the present inventors enforced KIAA0101 expression in NIH3T3 and investigated whether KIAA0101 overexpression transforms NIH3T3 *in vivo*. As shown in Fig.11C, three clones of NIH3T3-KIAA0101 formed the tumors at the right flank of nude mice), while NIH3T3-Mock did not
10 at the left flank, and the immunohistochemical staining of these tumors showed positive staining of KIAA0101 at the nucleus of tumor cells (Fig. 11D). These results implicated that KIAA0101 had its ability to transform normal cells to tumor cells.

Example 8

Identification of PCNA, POLD1, FEN1 as a complex of KIAA0101 protein.

15 To investigate the biological functions of KIAA0101 further, the present inventors carried out immunoprecipitation to identify complexes with KIAA0101protein, using a polyclonal antibody to KIAA0101. Silver-stained immunoprecipitated fractions separated on SDS-PAGE gels showed that several proteins were immunoprecipitated with KIAA0101 proteins from cancer cell lysate, compared with results from a control sample (Fig.12A).
20 Each protein was analyzed by a MALDI-TOF system after in-gel trypsin digestion; they were identified as PCNA, POLD1 (polymerase δ p125 subunit), and FEN1 (flap endonuclease-1). These interactions were confirmed by immunoprecipitation experiment shown Fig. 12B. All of these proteins are involved with DNA replication/repair, and POLD1 and FEN1 also bind to PCNA as well as KIAA0101 (Jonsson *et al.*, (1998) EMBO J. Apr 15;17(8):2412-25,
25 Zhang *et al.*, (1999) J Biol Chem. Sep 17;274(38):26647-53; Bruning & Shamoo (2004) Structure. Dec;12(12):2209-19.), suggesting that KIAA0101 can involve DNA replication through the interaction with PCNA.

Example 9

Inhibition of the interaction between KIAA0101 and PCNA by cell-permeable dominant-negative peptide.

30 To inhibit the interaction between KIAA0101 and PCNA through the conserved PIP box motif of KIAA0101, the present inventors designed the dominant-negative peptide

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containing this PIP box and conjugated this with arginine (R)-repeat to facilitate cell permeability (Fig. 13A). *In vitro* study, the present inventors validated the inhibition of the interaction between PCNA and KIAA0101 by immunoprecipitation. In the presence of PIP20, PCNA was not immunoprecipitated with KIAA0101, while PIP20mut where the conserved residues were replaced with alanine (Fig. 13A) and scramble peptide did not affect the interaction (Fig. 13B). Then the present inventors evaluated whether these peptides inhibited cancer cell growth by treating cancer cells and normal fibroblast NIH3T3 cell with these peptides. Mouse KIAA0101 (NP_080791) has high homology with human one and the target region for dominant-negative peptide in human KIAA0101 was 100% identical with that in mouse KIAA0101. Fig. 13C demonstrated that PIP20 suppressed cancer cell growth dose-dependently, while PIP20mut and scramble peptides did not. On the other hand, PIP20 did not affect the growth of mouse normal cell line NIH3T3 cells that did not express the homologue of human KIAA0101. These findings indicated that PIP20 specifically inhibited the cell growth by targeting KIAA0101.

Next the present inventors designed short PIP peptides by deleting some residues of N- and C-franking regions with PIP box motif maintained (PIP16 and PIP16mut shown in Fig. 13A) and treated cancer cell line and NIH3T3 cells with them. PIP16 treatment suppressed cancer cell growth strongly, however, PIP16 also affected NIH3T3 growth, and its growth-suppressive effect was lost by replacing the conserved residues of PIP box motif with alanines, as well as PIP20 (Fig. 13D). These findings suggested that the effect of PIP16 was not specific to KIAA0101 and PIP16 was likely to affect the interaction between PCNA and other DNA replication proteins.

Discussion

This invention demonstrated its potentiality as a molecular target for PDAC treatment. In order to invention the role or function of secreted REG4 in pancreatic carcinogenesis or progression, the present inventors knocked down the endogenous REG4 expression by siRNA in PDAC cell lines, and the present inventors exposed cancer cell with recombinant REG4. These findings from our experiments indicated that REG4 functions as an autocrine / paracrine growth factor and mediate Akt signaling pathways via unknown receptor. But it is unknown how REG4 mediate Akt signaling pathway, and that is the issue that should be investigated by further studies, in addition to identification of REG4 receptor (Kobayashi S, *et al.* J Biol Chem 2000; 275: 10723-6.).

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Recent researches reported that REG4 and other family member, REG1, were likely to function as anti-apoptotic factor for colon cancer or gastric cancer through Akt pathway (Sekikawa A, *et al.* Gastroenterology 2005; 128: 642-53., Bishnupuri KS, *et al.* Gastroenterology 2006; 130: 137-49.), and our study validated this activated pathways by treatment of secreted REG4 and Akt signaling pathway is most likely to be the downstream pathways of REG family signaling associated with cancer growth or anti-apoptosis. REG family is seemed to be expressed in the tissue injury or the regeneration process and to play some important roles of tissue regeneration (Unno M, *et al.* Adv Exp Med Biol 1992; 321: 61-6., Zhang YW, *et al.* World J Gastroenterol 2003; 9: 2635-41.). Considering that Akt signaling pathway activation by REG4 and other REG members, they may be associated with the 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 neutralizes secreted REG4 in the culture medium *in vitro* and treatment of these neutralizing antibodies significantly suppressed PDAC cell growth by shutting down REG4 autocrine/paracrine pathway and blocking the subsequent Akt phosphorylation. These findings implicate the feasibility of neutralizing antibody therapy targeting REG4. Bevacizumab, a humanized monoclonal antibody to 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 HGF (Burgess T, *et al.* Cancer Res. 2006; 66: 1721-9.) and IL-6 (Tripathi M, *et al.* Clin Cancer Res 2003; 9: 4653-65.), are under review as anti-cancer drugs, and neutralizing-antibody therapy targeting REG4 may also provide us with a novel therapeutic strategy for PDACs and other cancer expressing REG4.

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

Moreover, the present inventors identified one over-expressing gene, KIAA0101, in pancreatic cancer cells. In RNA level, according to the information of the present inventor's

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microarray analysis on several human cancers and some reports (Peiwen *et al.*, (2001) Oncogene, 20: 484-9; Mizutani *et al.*, (2005) Cancer, 103: 1785-90), many other cancer cells also over-expressed KIAA0101 and its expression is observed generally in highly proliferating cells. Immunohistochemical analysis using anti-KIAA0101 antibody showed that KIAA0101 was highly expressed in cancer cell and in some level at the crypt of normal intestinal mucosa and the germinal center of lymph-node where proliferating cells are present. And KIAA0101 expression was dependent on cell cycle and its expression was highest in S phase, at which DNA replication is most active (data not shown), which strongly implicates that KIAA0101 expression is involved with cell proliferation. Indeed knockdown of KIAA0101 by siRNA suppressed cell proliferation in cancer cells in this invention, and previous report identified KIAA0101 as a PCNA-binding protein by yeast two-hybrid system (Yu *et al.*, (2001) Oncogene, 20: 484-9).

PCNA is an essential auxiliary protein for the processes of DNA replication and DNA repair and acting as a clamp platform that slides along the DNA template, interacting with numerous DNA synthesis or metabolic enzymes (Wyman and Botchan, (1995) Curr Biol., 5: 334-7; Warbrick, (2000) Bioessays, 22: 997-1006; Krishna *et al.*, (1994) Cell, 79: 1233-43). The present inventors here show that KIAA0101 also binds to PCNA directly through its conserved PIP box motif and KIAA0101 is likely to coordinate PCNA function by binding with PCNA or competing with other PCNA-binding partners such as p21 (Waga *et al.*, (1994) Nature 369: 574-8; Chen *et al.*, (1996) Nucl Acid Res 24: 1727-33; Kontopidis *et al.*, (2005) PNAS, 102: 1871-6). In the present invention, the present inventors focused on this PIP box motif and designed the dominant-negative peptide conjugating with cell permeable arginine-repeat (Noguchi *et al.*, (2004) Nat Med., 10: 305-9) to inhibit the interaction between PCNA and KIAA0101 specifically. PIP20 suppressed cancer cell growth dose-dependently, while PIP20mut did not, and PIP20 did not affect the growth of NIH3T3 which did not expressed KIAA0101, suggesting its high specificity.

Interestingly, the present inventors deleted some residues at the flanking regions of PIP20, maintaining PIP box, to design the shorter peptide PIP16 and PIP16, which more strongly suppressed cancer cell growth than PIP20, but it seemed to lose its specificity to KIAA0101-PCNA interaction. PCNA are interacting with numerous proteins through their PIP box motif (Wyman and Botchan, (1995) Curr Biol., 5: 334-7; Warbrick, (2000) Bioessays, 22: 195-1006; Krishna *et al.*, (1994) Cell, 79: 1233-43; Chen *et al.*, (1996) Nucl Acid Res 24: 1727-33; Kontopidis *et al.* (2005) PNAS, 102: 1871-6) and it is possible that PIP16 can

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inhibit the interaction between PCNA and other many proteins that play essential roles of cell proliferation and are ubiquitously expressed. The flanking residues of PIP20 are likely to be important in specific inhibition between KIAA0101 and PCNA. In the similar way, the peptides derived from p21 PIP box, which also can interact with PCNA, are capable of
5 arresting and killing cancer cell and inhibiting of PCNA-dependent DNA replication can be a promising strategy for cancer strategy (Chen *et al.*, (1996) Nucl Acid Res 24: 1727-33; Kontopidis *et al.* (2005) PNAS, 102: 1871-6).

Intracellular protein-protein interactions constitute major control points in many signaling pathways, yet have frequently proven a difficult target for small molecule chemistry,
10 often reflecting a protein interface that is extensive, shallow, and hydrophobic (Walensky *et al.*, (2004) Science, 305: 1446-70). Although peptides are attractive candidates for stabilizing or disrupting protein-protein interactions, their efficacy as *in vivo* reagents is severely compromised by their loss of secondary structure, susceptibility to proteolytic degradation, and difficulty in penetrating intact cells. But a recent report about the
15 modification of peptides indicated its feasibility as druggable targets (Walensky *et al.*, (2004) Science, 305: 1446-70), and further structural analysis of targeted protein-protein interaction (Kontopidis *et al.*, (2005) PNAS, 102: 1871-6) or DDS improvement can provide the peptide inhibiting protein-protein interaction with more attraction for drug development and promising feasibility for drug development.

20 **Industrial Applicability:**

The present inventors have shown that the cell growth is suppressed by small interfering RNA (siRNA) that specifically target the REG4 gene or KIAA0101 gene. Thus, this novel siRNAs are useful target for the development of anti-cancer pharmaceuticals. For example, agents that block the expression of REG4 or prevent its activity may find therapeutic
25 utility as anti-cancer agents, particularly anti-cancer agents for the treatment of pancreatic cancer, such as pancreatic ductal adenocarcinoma (PDAC). Similarly, agents that block the expression of KIAA0101 or prevent its activity may find therapeutic utility as anticancer agents for the treatment of pancreatic cancer, prostatic cancer, breast cancer, and bladder cancer.

30 The present inventors also have shown that a monoclonal antibody against REG4 neutralized its growth-promoting effects and attenuated significantly the growth of PDAC cells. Thus, treatment of disease associated with REG4-expressing cells, for example, pancreatic cancer is conveniently carried out using antibodies that bind to REG4.

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The present inventors also have shown that KIAA0101 interacts with PCNA, and the inhibition of the interaction leads to inhibition of cell proliferation of cancer cells. Thus, agents that inhibit the binding between KIAA0101 interacts with PCNA and prevent its activity have therapeutic utility as anti-cancer agents.

5 The present invention thus provides novel polypeptides and other compounds useful in treating or preventing cancer. The polypeptides of the present invention are composed of an amino acid sequence which contains QKGIGEFF/SEQ ID NO: 46. The polypeptides of the present invention can be administered to inhibit the proliferation of, or to induce apoptosis in, cancer cells. The polypeptides of the present invention are expected to exhibit cell
10 proliferation inhibiting effects against various cancers. Particularly, the polypeptides of the present invention have been confirmed to have cell proliferation inhibiting effects on pancreatic cancer.

Pancreatic cancer is an important cancer for which an effective treatment method is still desired to be provided. Therefore, the present invention is significant in that it also
15 provides an effective method for treating and/or preventing pancreatic cancer.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope of the invention.

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CLAIMS

1. A method for treating or preventing pancreatic cancer in a subject comprising administering to said subject a composition comprising at least one small interfering RNA (siRNA) that inhibits expression of REG4 or KIAA0101.
- 5 2. The method of claim 1, wherein said siRNA comprises a sense nucleic acid sequence and an anti-sense nucleic acid sequence that specifically hybridizes to a sequence from REG4 or KIAA0101.
3. The method of claim 1, wherein the cancer to be treated is selected from the group consisting of pancreatic cancer, prostatic cancer, breast cancer, and bladder cancer.
- 10 4. The method of claim 3, wherein the pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC).
5. The method of claim 1, wherein the siRNA inhibits expression of REG4 and the cancer to be treated is PDAC.
6. The method of claim 1, wherein the siRNA inhibits expression of KIAA0101 and the
15 cancer to be treated is pancreatic cancer, prostatic cancer, breast cancer, and bladder cancer.
7. The method of claim 2, wherein said siRNA comprises a ribonucleotide sequence corresponding to a sequence of SEQ ID NO: 5 or SEQ ID NO: 32 as the target sequence.
8. The method of claim 7, wherein said siRNA has the general formula 5'-[A]-[B]-[A']-3',
20 wherein [A] is a ribonucleotide sequence corresponding to a sequence of nucleotides of SEQ ID NO: 5 or SEQ ID NO: 32.
[B] is a ribonucleotide loop sequence consisting of 3 to 23 nucleotides, and
[A'] is a ribonucleotide sequence consisting of the complementary sequence of [A].
9. A double-stranded molecule comprising a sense strand and an antisense strand, wherein
25 the sense strand comprises a ribonucleotide sequence corresponding to a target sequence of SEQ ID NO: 5 or SEQ ID NO: 32, and wherein the antisense strand comprises a ribonucleotide sequence which is complementary to said sense strand, wherein said sense strand and said antisense strand hybridize to each other to form said double-stranded molecule, and wherein said double-stranded molecule, when introduced into a cell
30 expressing the REG4 or KIAA0101 gene, inhibits expression of said gene.

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10. The double-stranded molecule of claim 9, wherein said target sequence comprises at least about 10 contiguous nucleotides from the nucleotide sequences of SEQ ID NO: 1 or SEQ ID NO: 39.
11. The double-stranded molecule of claim 10, wherein said target sequence comprises from
5 about 19 to about 25 contiguous nucleotides from the nucleotide sequences of SEQ ID NO: 1 or SEQ ID NO: 39.
12. The double-stranded molecule of claim 11, wherein said double-stranded molecule is a single ribonucleotide transcript comprising the sense strand and the antisense strand linked via a single-stranded ribonucleotide sequence.
- 10 13. The double-stranded molecule of claim 10, wherein the double-stranded molecule is an oligonucleotide of less than about 100 nucleotides in length.
14. The double-stranded molecule of claim 13, wherein the double-stranded molecule is an oligonucleotide of less than about 75 nucleotides in length.
15. The double-stranded molecule of claim 14, wherein the double-stranded molecule is an
15 oligonucleotide of less than about 50 nucleotides in length.
16. The double-stranded molecule of claim 15, wherein the double-stranded molecule is an oligonucleotide of less than about 25 nucleotides in length.
17. The double-stranded polynucleotide of claim 16, wherein the double stranded molecule is an oligonucleotide of between about 19 and about 25 nucleotides in length.
- 20 18. A vector encoding the double-stranded molecule of claim 10.
19. The vector of claim 18, wherein the vector encodes a transcript having a secondary structure and comprises the sense strand and the antisense strand.
20. The vector of claim 19, wherein the transcript further comprises a single-stranded ribonucleotide sequence linking said sense strand and said antisense strand.
- 25 21. A vector comprising a polynucleotide comprising a combination of a sense strand nucleic acid and an antisense strand nucleic acid, wherein said sense strand nucleic acid comprises nucleotide sequence of SEQ ID NO: 5 or SEQ ID NO: 32, and said antisense strand nucleic acid consists of a sequence complementary to the sense strand.
22. The vector of claim 21, wherein said polynucleotide has the general formula
30 5'-[A]-[B]-[A']-3',

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wherein [A] is a nucleotide sequence of SEQ ID NO: 5 or SEQ ID NO: 32;
[B] is a nucleotide sequence consisting of 3 to 23 nucleotides; and
[A'] is a nucleotide sequence complementary to [A].

23. A pharmaceutical composition for treating or preventing pancreatic cancer comprising a
5 pharmaceutically effective amount of a small interfering RNA (siRNA) that inhibits
expression of REG4 as an active ingredient, and a pharmaceutically acceptable carrier.

24. The pharmaceutical composition of claim 23, wherein the siRNA comprises a nucleotide
sequence of SEQ ID NO: 5 as the target sequence.

25. The composition of claim 24, wherein the siRNA has the general formula

10 5'-[A]-[B]-[A']-3'

wherein [A] is a ribonucleotide sequence corresponding to a nucleotide sequence of SEQ
ID NO: 5;

[B] is a ribonucleotide sequence consisting of 3 to 23 nucleotides; and

[A'] is a ribonucleotide sequence complementary to [A].

15 26. A pharmaceutical composition for treating or preventing pancreatic cancer, prostatic
cancer, breast cancer, and bladder cancer, comprising a pharmaceutically effective amount
of a small interfering RNA (siRNA) that inhibits expression of KIAA0101 as an active
ingredient, and a pharmaceutically acceptable carrier.

27. The pharmaceutical composition of claim 26, wherein the siRNA comprises a nucleotide
20 sequence of SEQ ID NO: 32 as the target sequence.

28. The composition of claim 27, wherein the siRNA has the general formula

5'-[A]-[B]-[A']-3'

wherein [A] is a ribonucleotide sequence corresponding to a nucleotide sequence of SEQ
ID NO: 32;

25 [B] is a ribonucleotide sequence consisting of 3 to 23 nucleotides; and

[A'] is a ribonucleotide sequence complementary to [A].

29. Use of a small interfering RNA (siRNA) that inhibits expression of REG4 for
manufacturing a pharmaceutical composition for treating or preventing pancreatic cancer.

30. Use of claim 29, wherein the siRNA comprises a nucleotide sequence of SEQ ID NO: 5
30 as the target sequence.

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31. Use of a small interfering RNA (siRNA) that inhibits expression of KIAA0101 for manufacturing a pharmaceutical composition for treating or preventing pancreatic cancer, prostatic cancer, breast cancer, and bladder cancer.
32. Use of claim 31, wherein the siRNA comprises a nucleotide sequence of SEQ ID NO: 32 as the target sequence.
33. A method for treating or preventing pancreatic cancer in a subject comprising administering to said subject an anti-REG4 antibody or fragment thereof that neutralizes REG4 activity.
34. The method of claim 33, wherein the REG4 activity to be neutralized is an activity to promote the cell proliferation of pancreatic cancer in autocrine/paracrine manner.
35. The method of claim 33, wherein the pancreatic cancer is a pancreatic ductal adenocarcinoma (PDAC).
36. The method of claim 33, wherein the anti- REG4 antibody is a monoclonal antibody.
37. The method of claim 33, wherein the antibody comprises a VH and VL chain, each VH and VL chain comprising CDR amino acid sequences designated CDR1, CDR2 and CDR3 separated by framework amino acid sequences, the amino acid sequence of each CDR in each VH and VL chain is:
- VH CDR1 : SYWMH (SEQ ID NO: 20),
VH CDR2 : NIYPGSGSTNYD (SEQ ID NO: 21),
VH CDR3 : GGLWLRVDY (SEQ ID NO: 22),
VL CDR1 : SASSSVSYMH (SEQ ID NO: 23),
VL CDR2 : DTSKLAS (SEQ ID NO: 24), and
VL CDR3 : QQWSSNPF (SEQ ID NO: 25).
38. The method of claim 36, wherein the human VH comprises the amino acid sequence of SEQ ID NO: 18, and human VL comprises the amino acid sequence of SEQ ID NO: 19.
39. A pharmaceutical composition for treating or preventing pancreatic cancer, said composition comprising a pharmaceutically effective amount of an anti-REG4 antibody or fragment thereof that neutralizes REG4 activity as an active ingredient, and a pharmaceutically acceptable carrier.
40. The pharmaceutical composition of claim 39, wherein the pancreatic cancer is a

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pancreatic ductal adenocarcinoma (PDAC).

41. The pharmaceutical composition of claim 39, wherein the anti-REG4 antibody is a monoclonal antibody.

42. The pharmaceutical composition of claim 39, wherein the antibody comprises a VH and VL chain, each VH and VL chain comprising CDR amino acid sequences designated CDR1, CDR2 and CDR3 separated by framework amino acid sequences, the amino acid sequence of each CDR in each VH and VL chain is:

VH CDR1 : SYWMH (SEQ ID NO: 20),

VH CDR2 : NIYPGSGSTNYD (SEQ ID NO: 21),

VH CDR3 : GGLWLRVDY (SEQ ID NO: 22),

VL CDR1 : SASSSVSYM (SEQ ID NO: 23),

VL CDR2 : DTSKLAS (SEQ ID NO: 24), and

VL CDR3 : QQWSSNPF (SEQ ID NO: 25).

43. The composition of claim 42, wherein the human VH comprises the amino acid sequence of SEQ ID NO: 18, and human VL comprises the amino acid sequence of SEQ ID NO: 19.

44. An antibody comprises a VH and VL chain, each VH and VL chain comprising CDR amino acid sequences designated CDR1, CDR2 and CDR3 separated by framework amino acid sequences, the amino acid sequence of each CDR in each VH and VL chain is:

VH CDR1 : SYWMH (SEQ ID NO: 20),

VH CDR2 : NIYPGSGSTNYD (SEQ ID NO: 21),

VH CDR3 : GGLWLRVDY (SEQ ID NO: 22),

VL CDR1 : SASSSVSYM (SEQ ID NO: 23),

VL CDR2 : DTSKLAS (SEQ ID NO: 24), and

VL CDR3 : QQWSSNPF (SEQ ID NO: 25).

45. The antibody of claim 44, wherein the human VH comprises the amino acid sequence of SEQ ID NO: 18, and human VL comprises the amino acid sequence of SEQ ID NO: 19.

46. An isolated polynucleotide encoding the antibody of claim 44.

47. A vector comprising a polynucleotide encoding the antibody of claim 44.

48. An isolated host cell comprising a vector comprising a polynucleotide encoding the antibody of claim 44.

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49. A process of producing an antibody comprising culturing the host cell of claim 48 so that the polynucleotide is expressed, and recovering the antibody from the host cell culture.

50. A pharmaceutical composition for treating or preventing pancreatic cancer, the composition comprising the polynucleotide encoding the antibody of claim 44, or a vector comprising same.

51. Use of an antibody or fragment thereof that binds to a protein encoded by REG4 for manufacturing a pharmaceutical composition for treating or preventing pancreatic cancer.

52. Use of claim 51, wherein the antibody comprises a VH and VL chain, each VH and VL chain comprising CDR amino acid sequences designated CDR1, CDR2 and CDR3 separated by framework amino acid sequences, the amino acid sequence of each CDR in each VH and VL chain is:

VH CDR1 : SYWMH (SEQ ID NO: 20),
VH CDR2 : NIYPGSGSTNYD (SEQ ID NO: 21),
VH CDR3 : GGLWLRVDY (SEQ ID NO: 22),
VL CDR1 : SASSSVSYMHI (SEQ ID NO: 23),
VL CDR2 : DTSKLAS (SEQ ID NO: 24), and
VL CDR3 : QQWSSNPF (SEQ ID NO: 25).

53. An agent for either or both of treating and preventing cancer comprising as an active ingredient a polypeptide which comprises QKGIGIEFF/SEQ ID NO: 46; a polypeptide functionally equivalent to the polypeptide; or polynucleotide encoding those polypeptides, wherein the polypeptide lacks the biological function of a peptide consisting of SEQ ID NO:40.

54. The agent of claim 53, wherein the biological function is cell proliferation activity.

55. The agent of claim 53, wherein the polypeptide consists of 8 to 30 residues.

56. The agent of claim 53, wherein the polypeptide comprises the amino acid sequence VRPTPKWQKGIGIEFFRLSPK/SEQ ID NO. 44 or TPKWQKGIGIEFFRLSP/SEQ ID NO. 45.

57. The agent of claim 53, wherein the polypeptide consists of the amino acid sequence VRPTPKWQKGIGIEFFRLSPK/SEQ ID NO. 44 or TPKWQKGIGIEFFRLSP/SEQ ID NO. 45.

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58. The agent of claim 53, wherein the active ingredient is the polypeptide and said polypeptide is modified with a cell-membrane permeable substance.

59. The agent of claim 58, wherein the polypeptide has the following general formula:
[R]-[D];

5 wherein [R] represents the cell-membrane permeable substance; and [D] represents the amino acid sequence of the fragment sequence which comprises QKGIGEFF/SEQ ID NO: 46; or a polypeptide functionally equivalent to the polypeptide, wherein the polypeptide lacks the biological function of a peptide consisting of SEQ ID NO:40.

60. The agent of claim 58, wherein the cell-membrane permeable substance is any one
10 selected from the group consisting of:

poly-arginine;

Tat / RKKRRQRRR/SEQ ID NO: 47;

Penetratin / RQIKIWFQNRRMKWKK/SEQ ID NO: 48;

Buforin II / TRSSRAGLQFPVGRVHRLLRK/SEQ ID NO: 49;

15 Transportan / GWTLNSAGYLLGKINLKALAALAKKIL/SEQ ID NO: 50;

MAP (model amphipathic peptide) / KLALKLALKALKAALKLA/SEQ ID NO: 51;

K-FGF / AAVALLPAVLLALLAP/SEQ ID NO: 52;

Ku70 / VPMLK/SEQ ID NO: 53 or PMLKE/SEQ ID NO: 61;

Prion / MANLGYWLLALFVTMWTDVGLCKKRPKP/SEQ ID NO: 54;

20 pVEC / LLILRRRIRKQAHASK/SEQ ID NO: 55;

Pep-1 / KETWWETWWTEWSQPKKKRKV/SEQ ID NO: 56;

SynB1 / RGGRLSYSRRRFSTSTGR/SEQ ID NO: 57;

Pep-7 / SDLWEMMMVSLACQY/SEQ ID NO: 58; and

HN-1 / TSPLNIHNGQKL/SEQ ID NO: 59.

25 61. The agent of claim 60, wherein the poly-arginine is Arg 11 (SEQ ID NO: 60).

62. The agent of claim 53, wherein the cancer is any one selected from the group consisting of pancreatic cancer, prostatic cancer, breast cancer, and bladder cancer.

63. A method for either or both of treating and preventing cancer comprising the step of administering a polypeptide comprising QKGIGEFF/SEQ ID NO: 46; a polypeptide
30 functionally equivalent to the polypeptide; or polynucleotide encoding these polypeptides, wherein the polypeptide lacks the biological function of a peptide consisting of SEQ ID NO: 40.

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64. Use of a polypeptide comprising QKGIGEFF/SEQ ID NO: 46; a polypeptide functionally equivalent to the polypeptide; or polynucleotide encoding those polypeptides in manufacturing a pharmaceutical composition for either or both of treating and preventing cancer, wherein the polypeptide lacks the biological function of a peptide consisting of SEQ ID NO: 40.
65. A pharmaceutical composition comprising a polypeptide comprising QKGIGEFF/SEQ ID NO: 46; or a polypeptide functionally equivalent to the polypeptide; and a pharmaceutically acceptable carrier, wherein the polypeptide lacks the biological function of a peptide consisting of SEQ ID NO: 40.
66. A polypeptide comprising QKGIGEFF/SEQ ID NO: 46; or an amino acid sequence of a polypeptide functionally equivalent to the polypeptide, wherein the polypeptide lacks the biological function of a peptide consisting of SEQ ID NO: 40.
67. The polypeptide of the claim 66, wherein the biological function is cell proliferation activity.
68. The polypeptide of claim 66, wherein the polypeptide consists of 8 to 30 residues.
69. The polypeptide of claim 66, wherein the polypeptide comprises the amino acid sequence VRPTPKWQKGIGEFFRLSPK/SEQ ID NO. 44 or TPKWQKGIGEFFRLSP/SEQ ID NO. 45.
70. The polypeptide of claim 66, wherein the polypeptide consists of the amino acid sequence VRPTPKWQKGIGEFFRLSPK/SEQ ID NO. 44 or TPKWQKGIGEFFRLSP/SEQ ID NO. 45.
71. The polypeptide of claim 66, wherein the polypeptide is modified with a cell-membrane permeable substance.
72. The polypeptide of claim 71, which has the following general formula:
[R]-[D];
wherein [R] represents the cell-membrane permeable substance; and [D] represents the amino acid sequence of a fragment sequence which comprises QKGIGEFF/SEQ ID NO: 46; or the amino acid sequence of a polypeptide functionally equivalent to the polypeptide comprising said fragment sequence, wherein the polypeptide lacks the biological function of a peptide consisting of SEQ ID NO: 40.

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73. The polypeptide of claim 72, wherein the cell-membrane permeable substance is any one selected from the group consisting of:

poly-arginine;

Tat / RKKRRQRRR/SEQ ID NO: 47;

5 Penetratin / RQIKIWFQNRRMKWKK/SEQ ID NO: 48;

Buforin II / TRSSRAGLQFPVGRVHRLLRK/SEQ ID NO: 49;

Transportan / GWTLNSAGYLLGKINLKALAALAKKIL/SEQ ID NO: 50;

MAP (model amphipathic peptide) / KLALKLALKALKAALKLA/SEQ ID NO: 51;

K-FGF / AAVALLPAVLLALLAP/SEQ ID NO: 52;

10 Ku70 / VPMLK/SEQ ID NO: 53

Ku70 / PMLKE/SEQ ID NO: 61;

Prion / MANLGYWLLALFVTMWTDVGLCKKRPKP/SEQ ID NO: 54;

pVEC / LLILRRRIRKQAAHAHSK/SEQ ID NO: 55;

Pep-1 / KETWWETWWTEWSQPKKKRKV/SEQ ID NO: 56;

15 SynB1 / RGGRLSYRRRFSTSTGR/SEQ ID NO: 57;

Pep-7 / SDLWEMMMVSLACQY/SEQ ID NO: 58; and

HN-1 / TSPLNIHNGQKL/SEQ ID NO: 59.

74. The polypeptide of claim 73, wherein the poly-arginine is Arg 11 (RRRRRRRRRRR/SEQ ID NO: 60).

20 75. A method for diagnosing the chemo-radiation therapeutic resistance of a cancer in a subject, which comprises the steps of:

(a) detecting the expression level of the *REG4* gene in a patient-derived biological sample;

(b) comparing the detected expression level to a control level; and

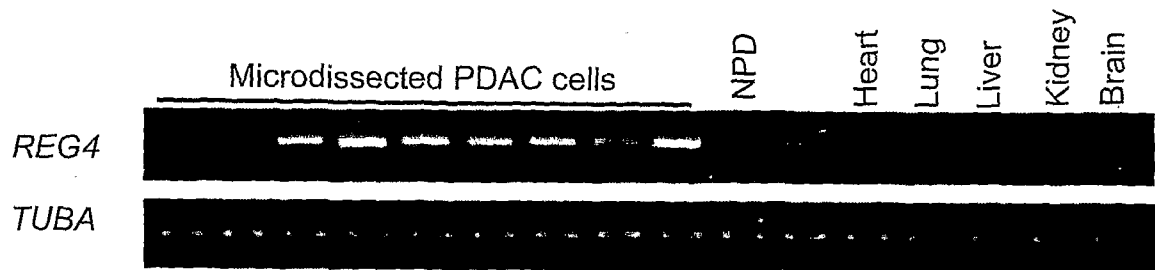
(c) determining the subject to suffer from the cancer of the chemo-radiation therapeutic
25 resistance or is at a risk of the chemo-radiation therapeutic resistance, when the detected expression level is increased to the normal control level.

76. The method of claim 75, wherein the cancer is pancreatic cancer.

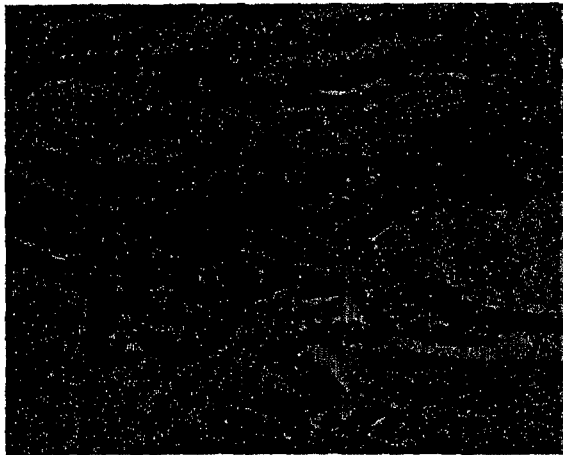
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Fig.1

A



B



C



D

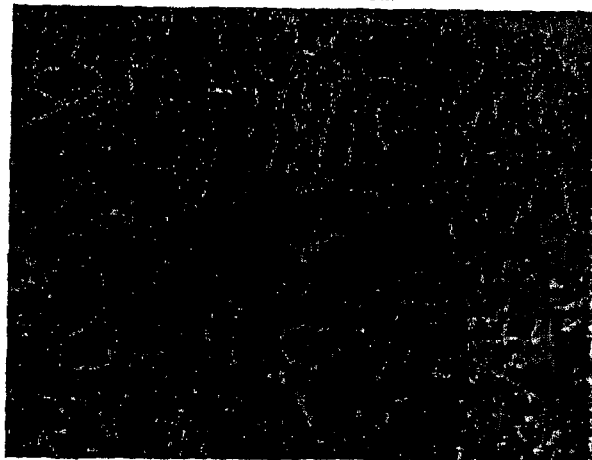
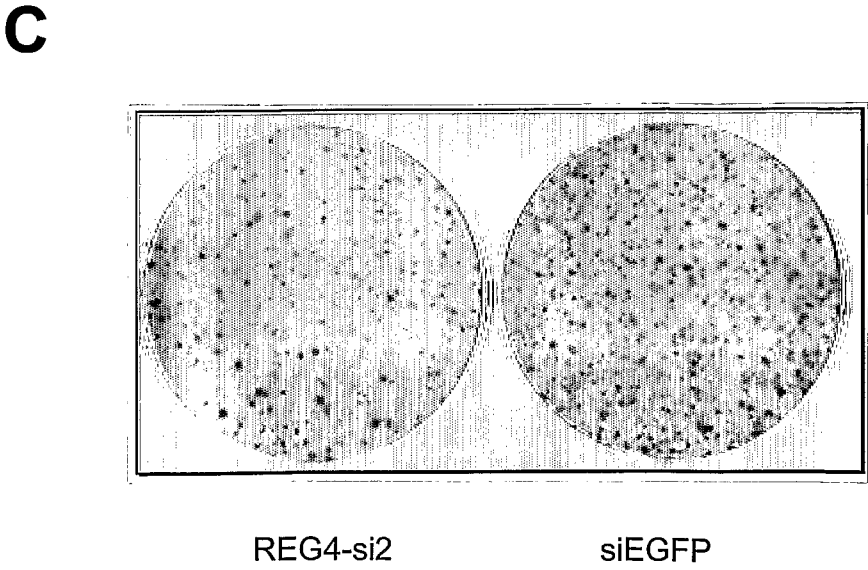
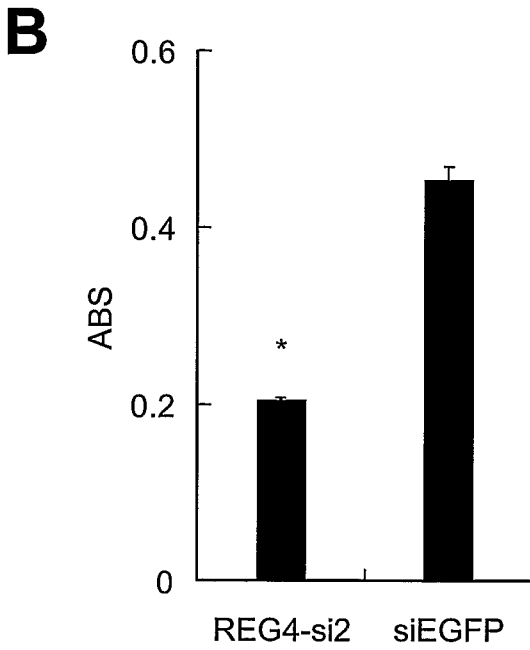
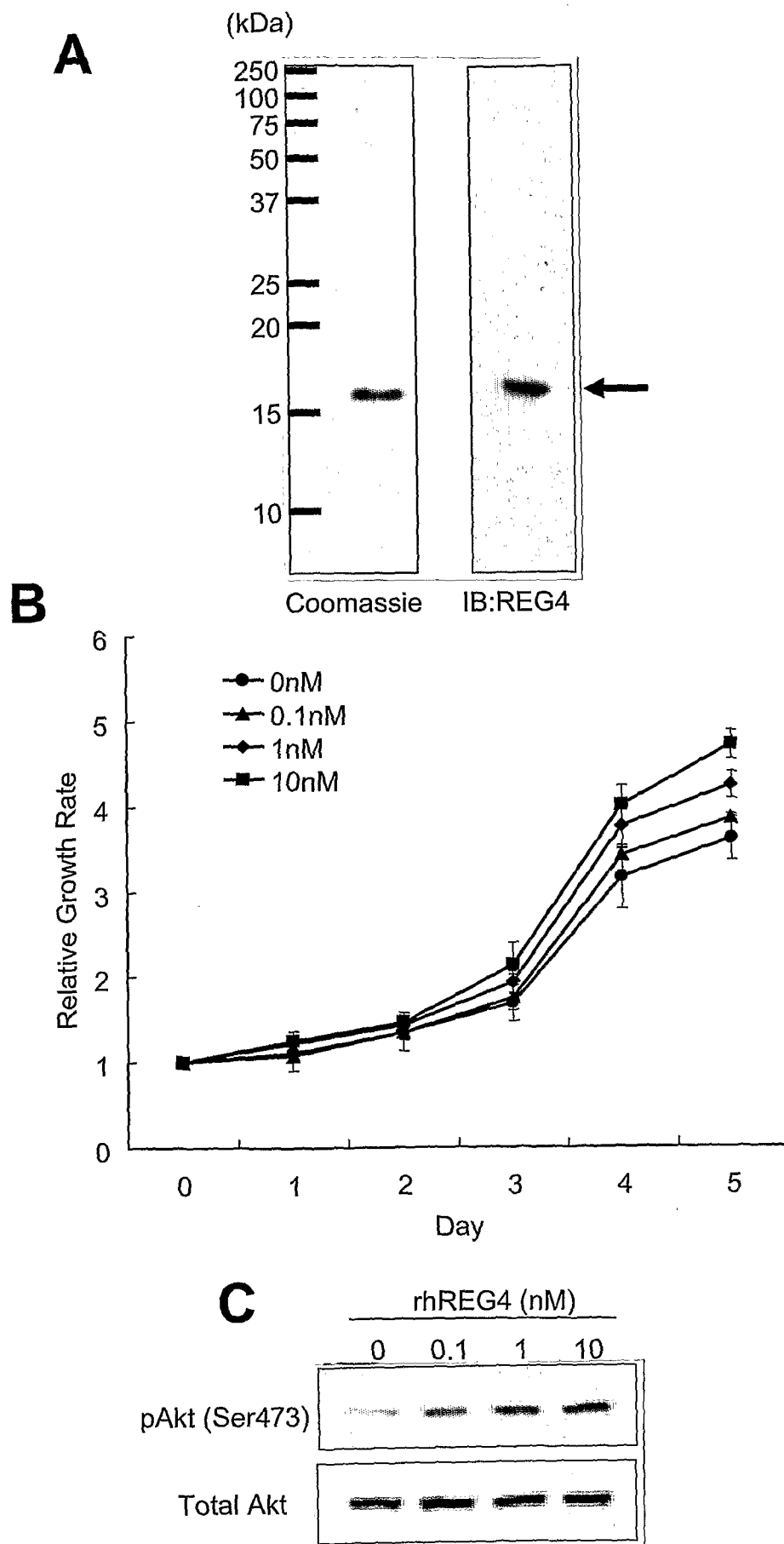


Fig.2



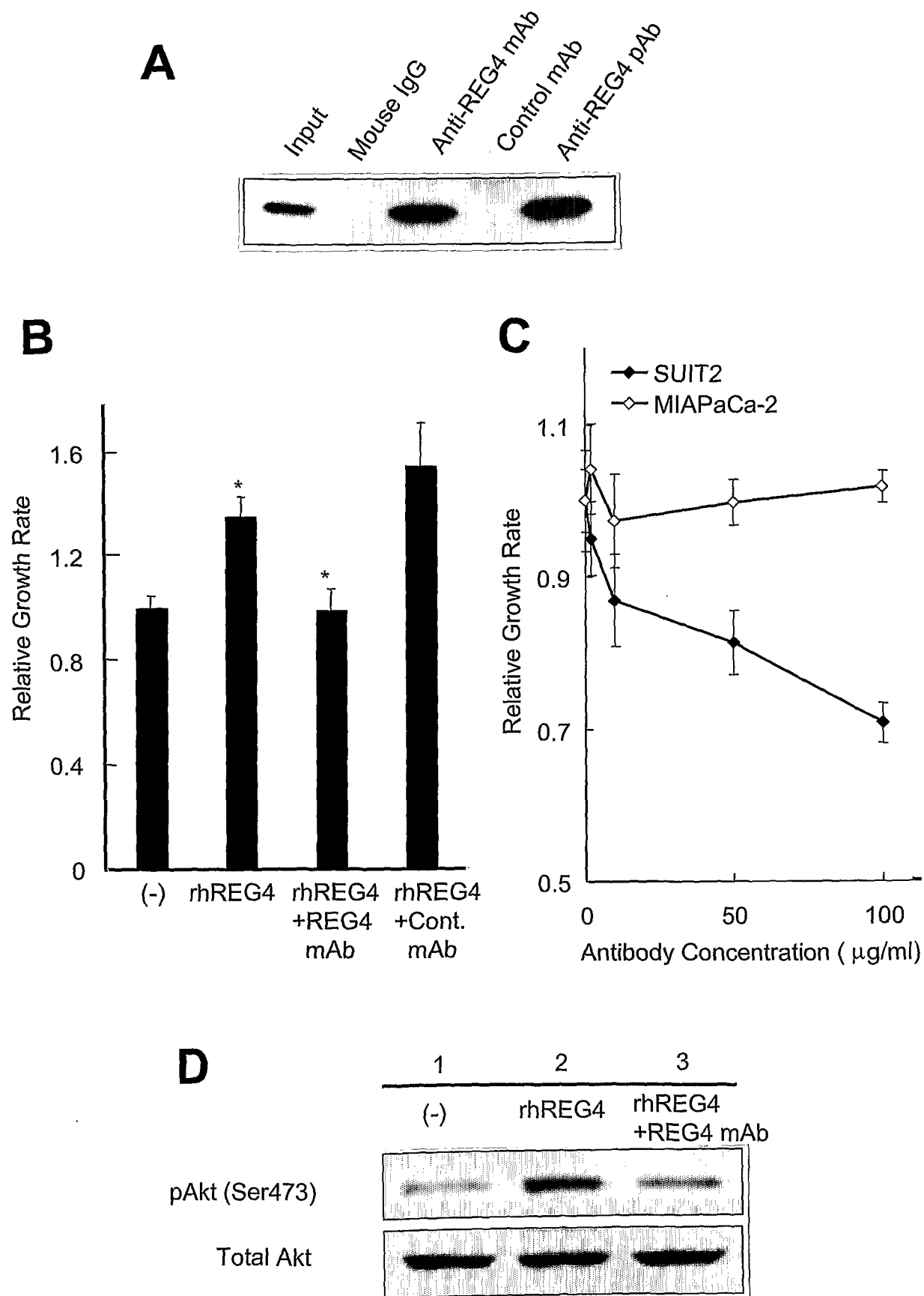
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Fig.3



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Fig.4



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Fig.5

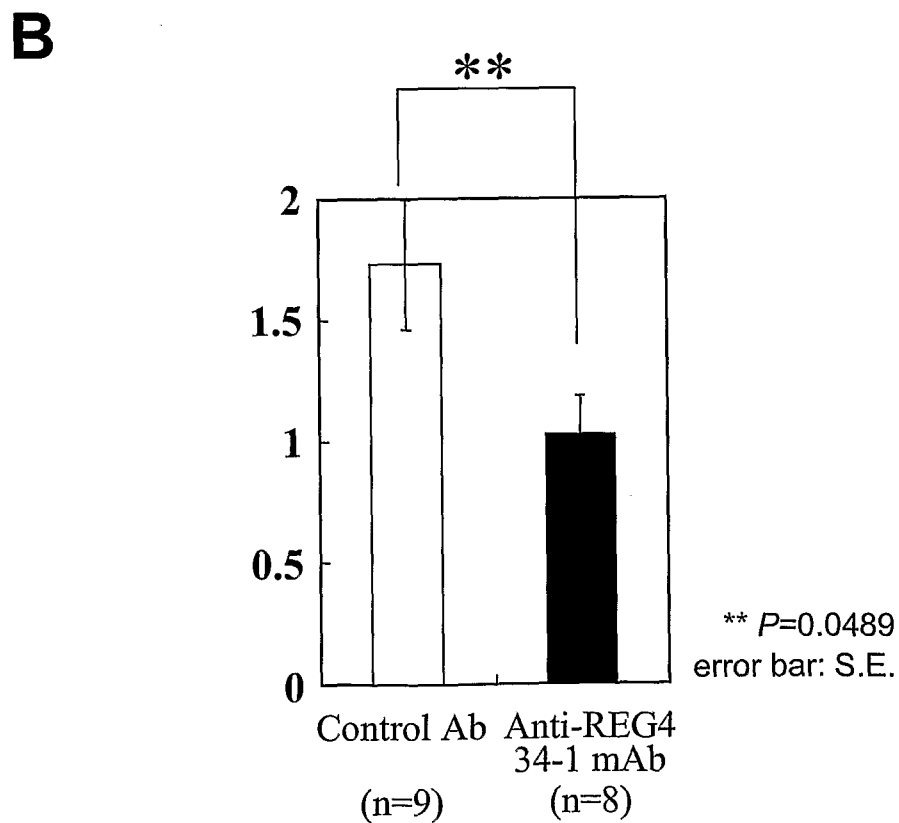
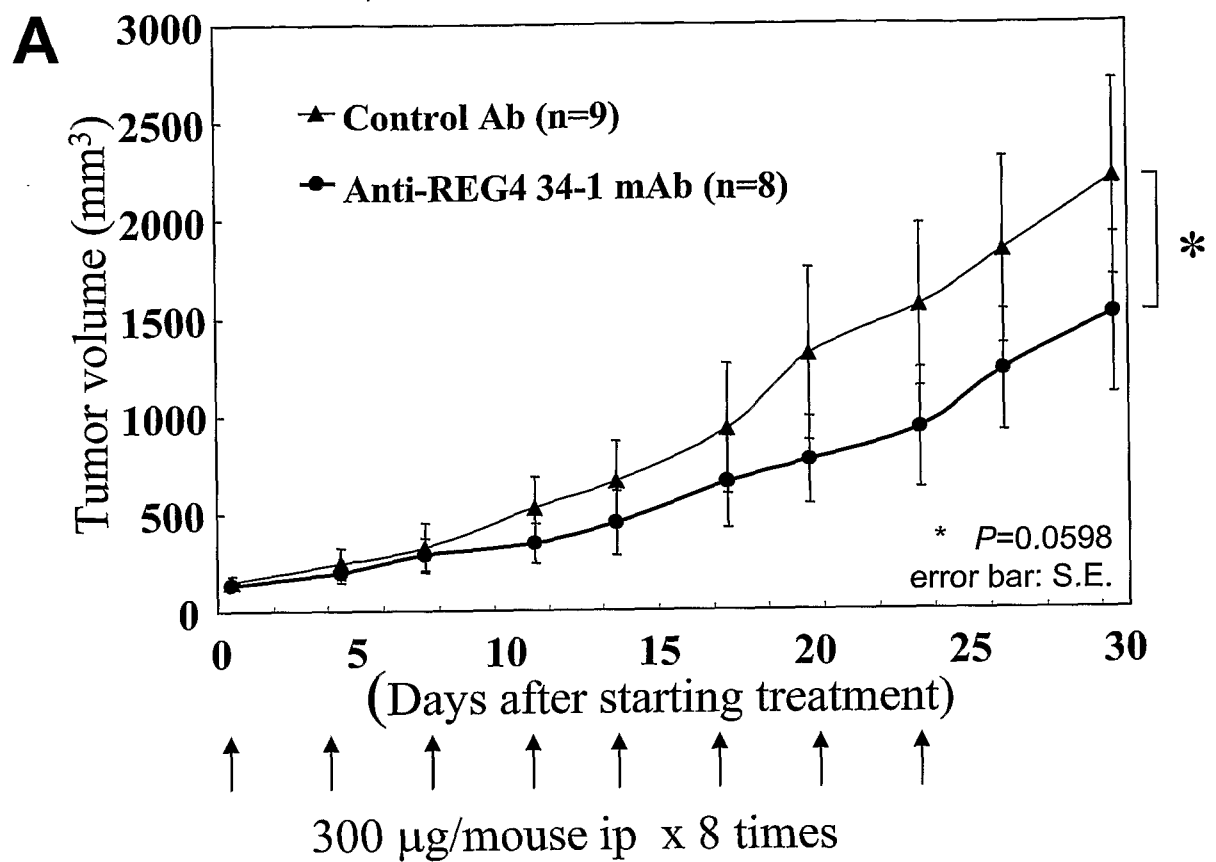
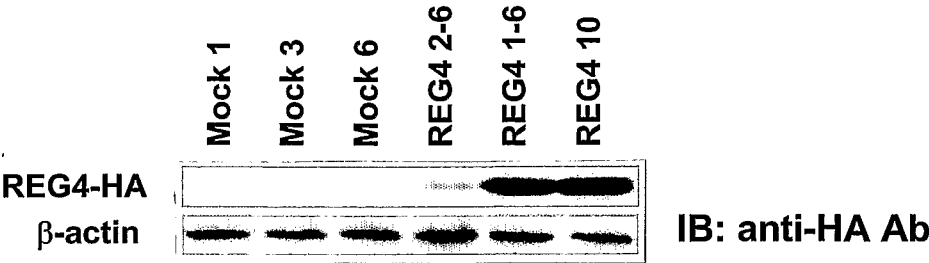
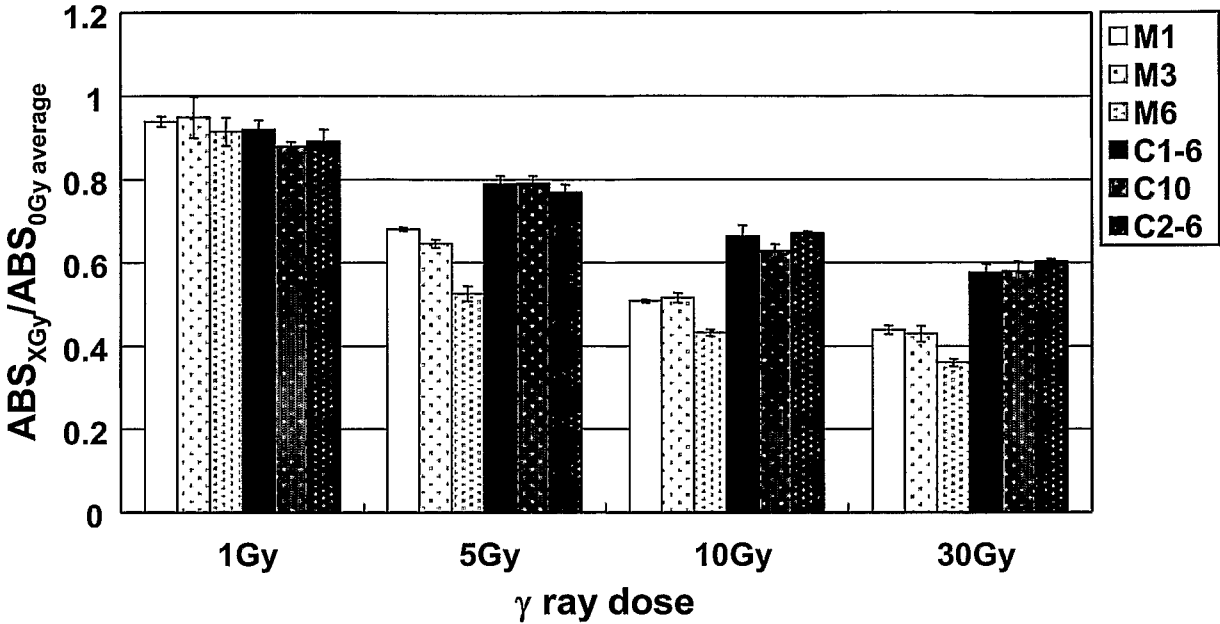


Fig.6

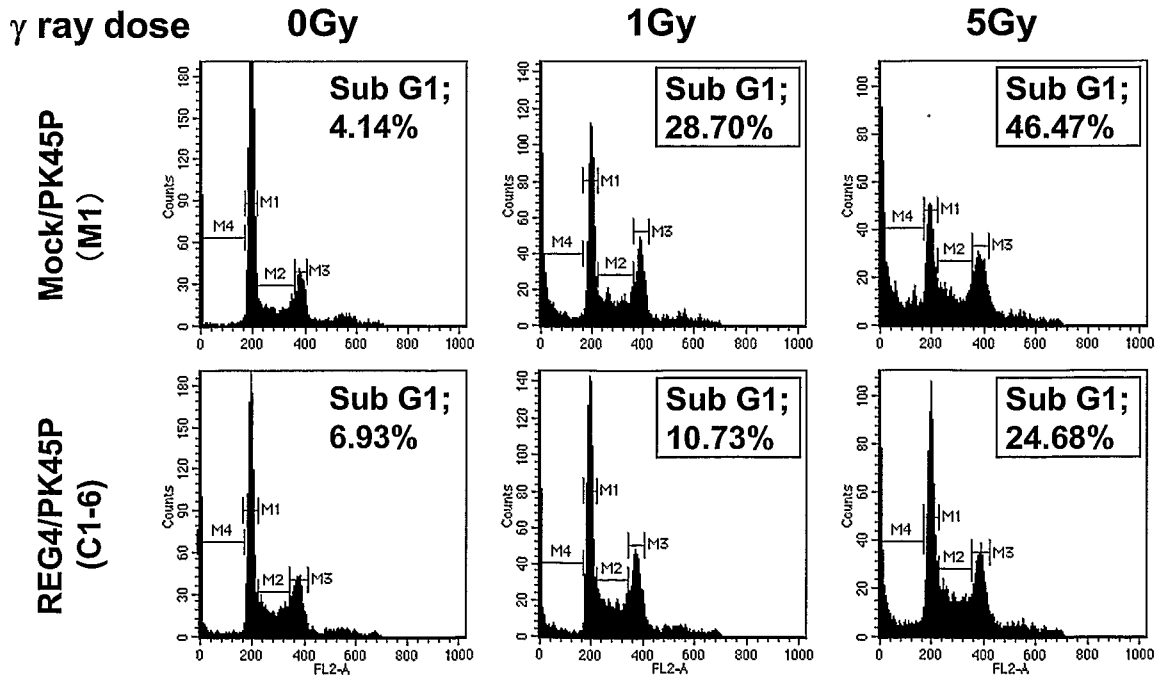
(A)



(B)



(C)

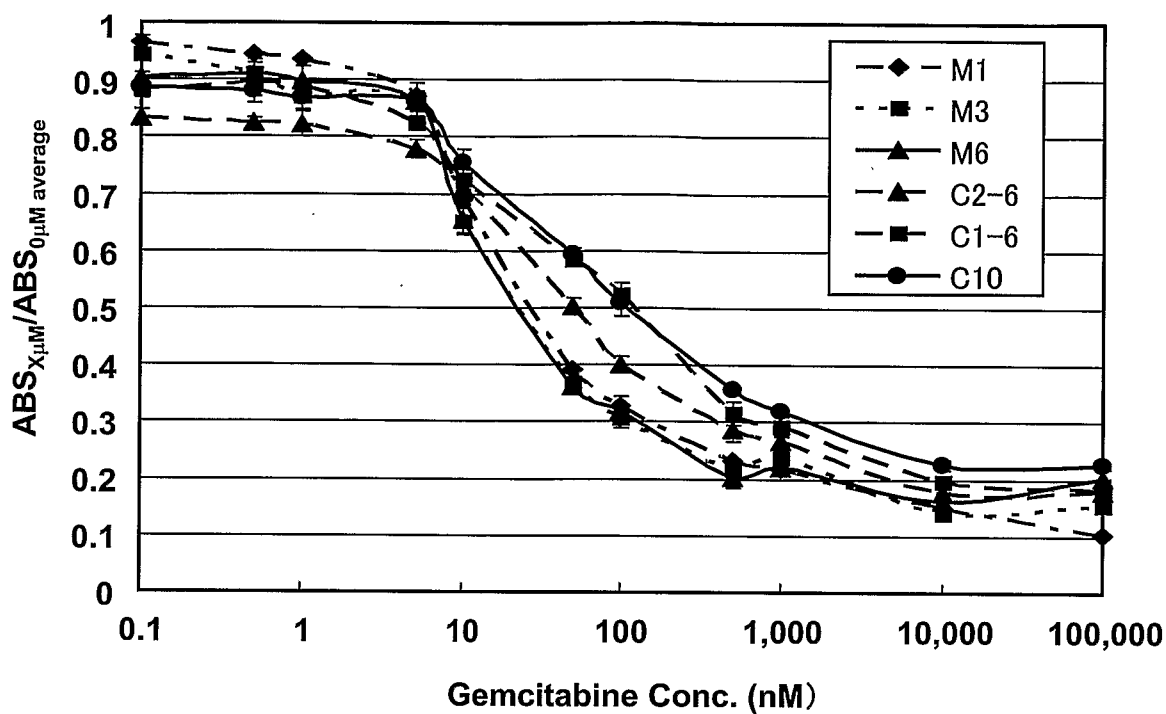


48,000 cells/well in 6well plate

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Fig.7

(A)



(B)

Gemcitabine Conc.

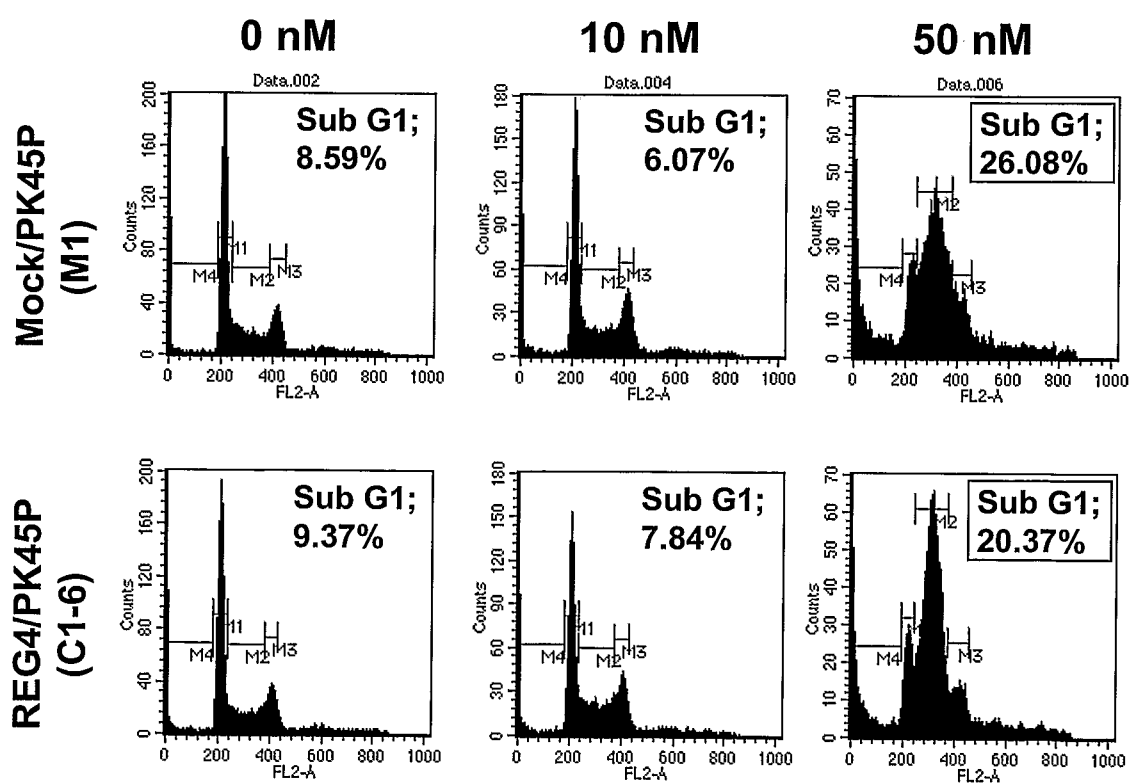
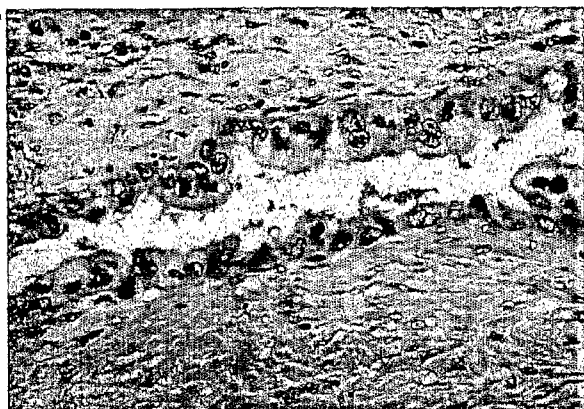
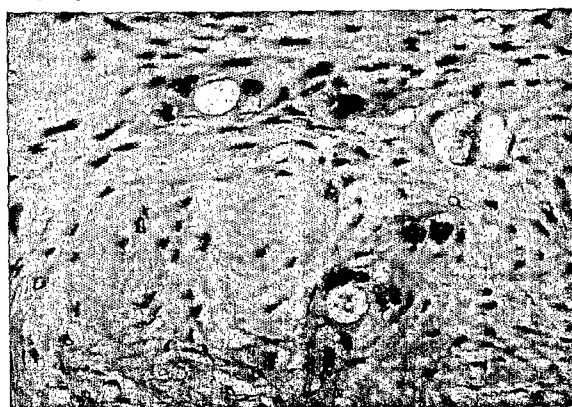


Fig.8

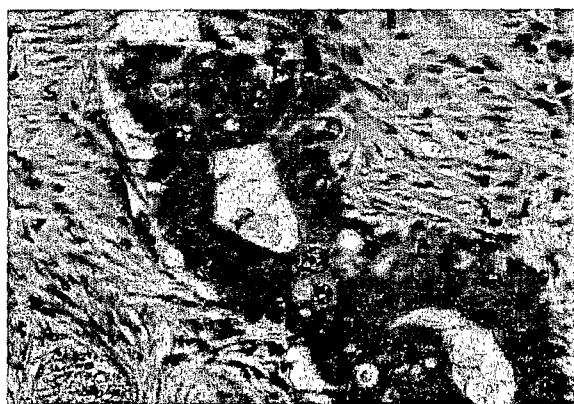
(A)



(B)



(C)



(D)

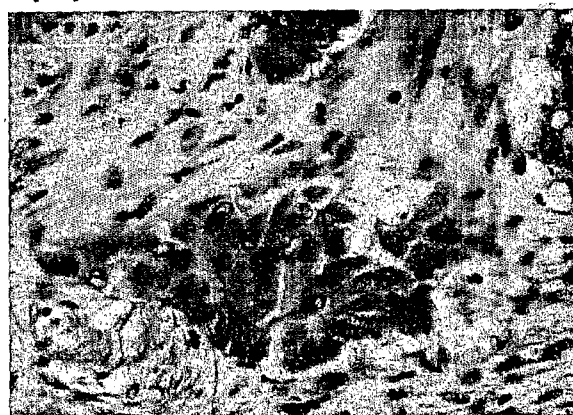
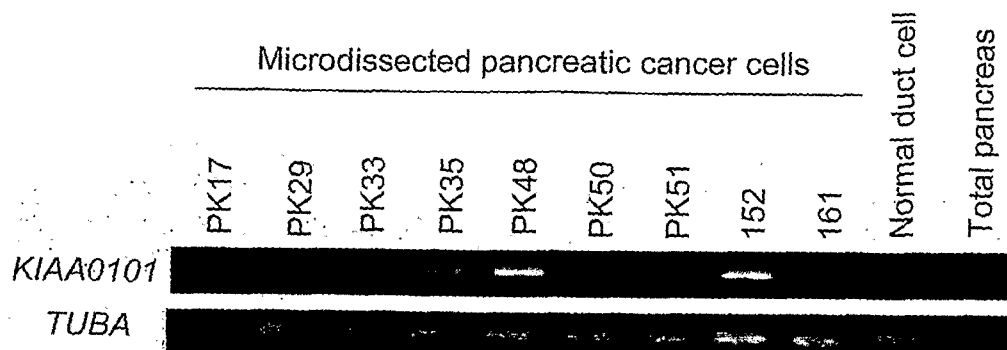
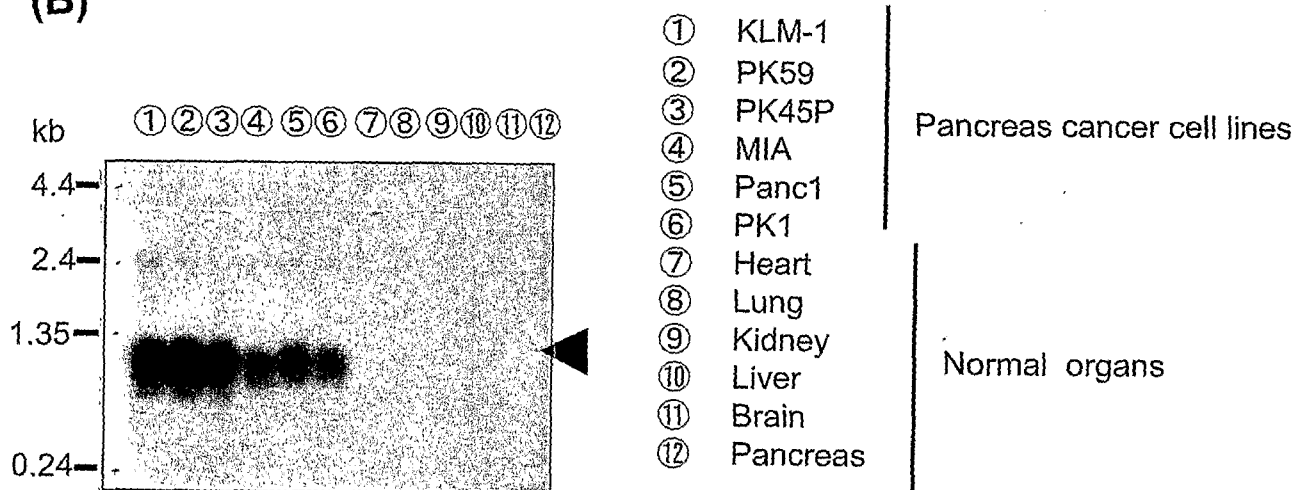


Fig.9

(A)



(B)



(C)



Pancreatic cancer x200



Pancreatic cancer x400

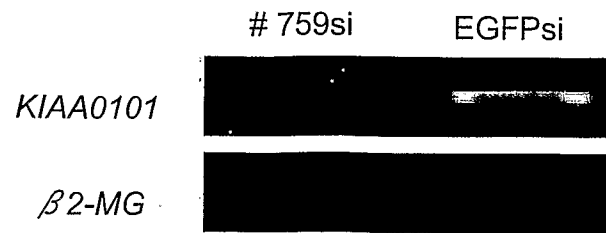


Normal pancreas x200

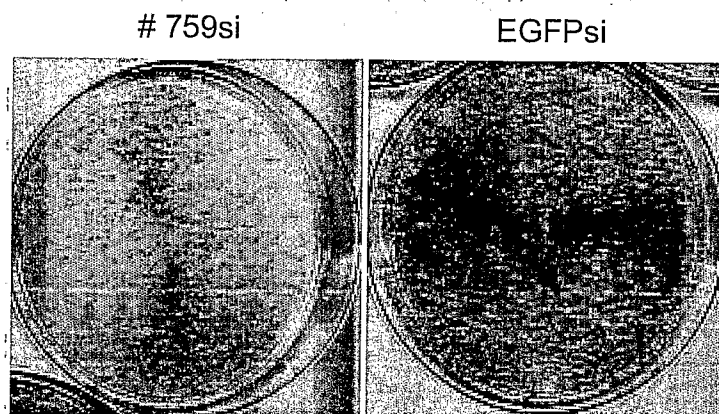
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Fig.10

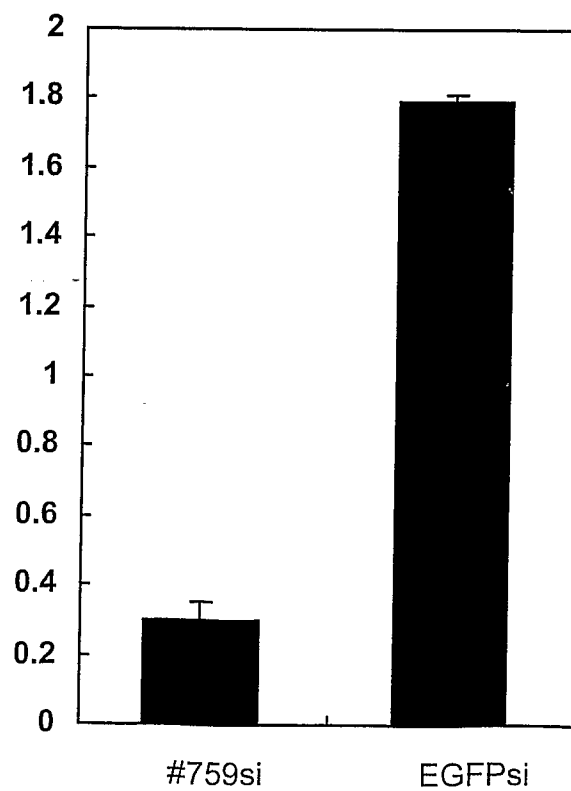
(A)



(B)

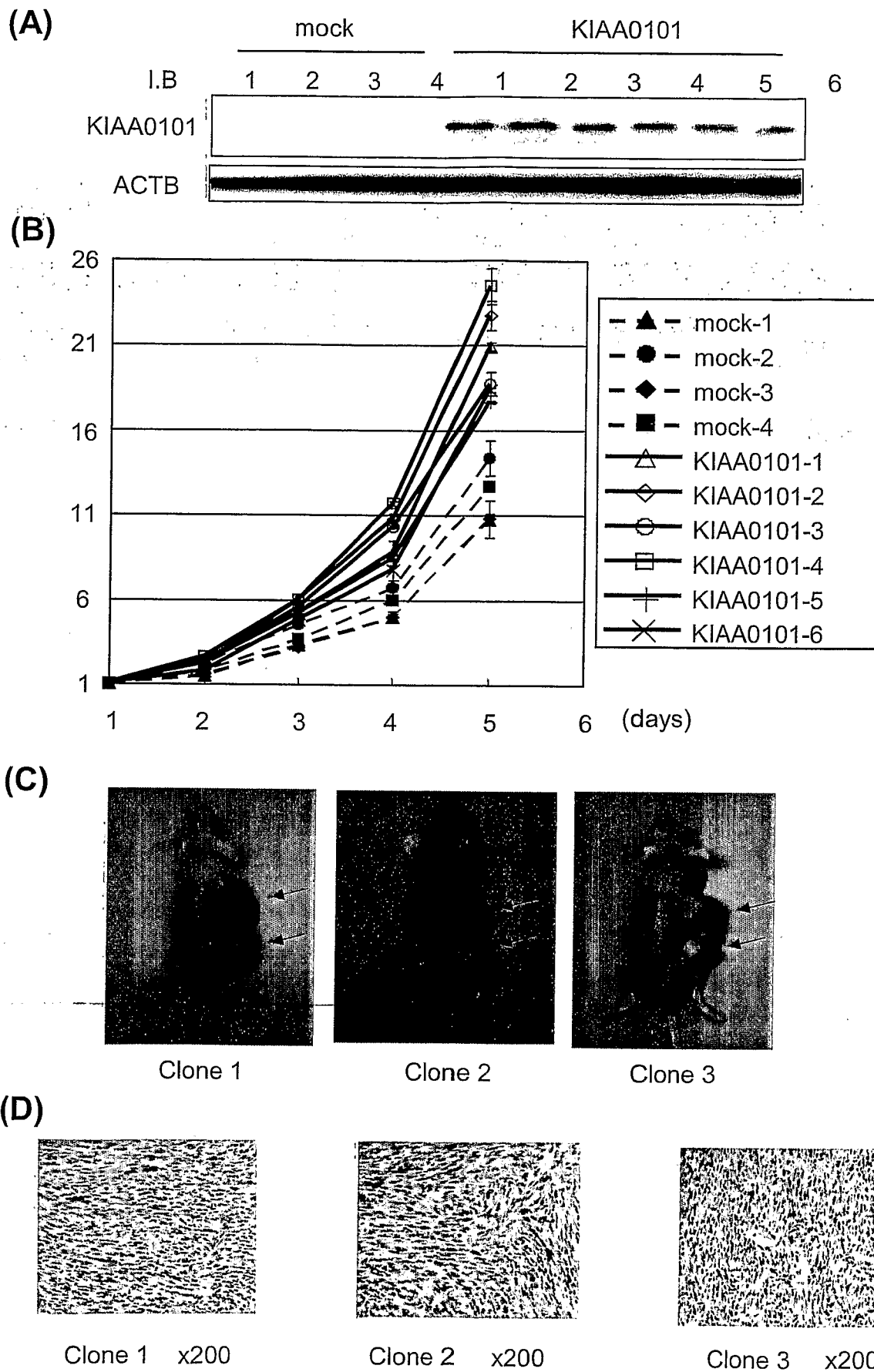


(C)



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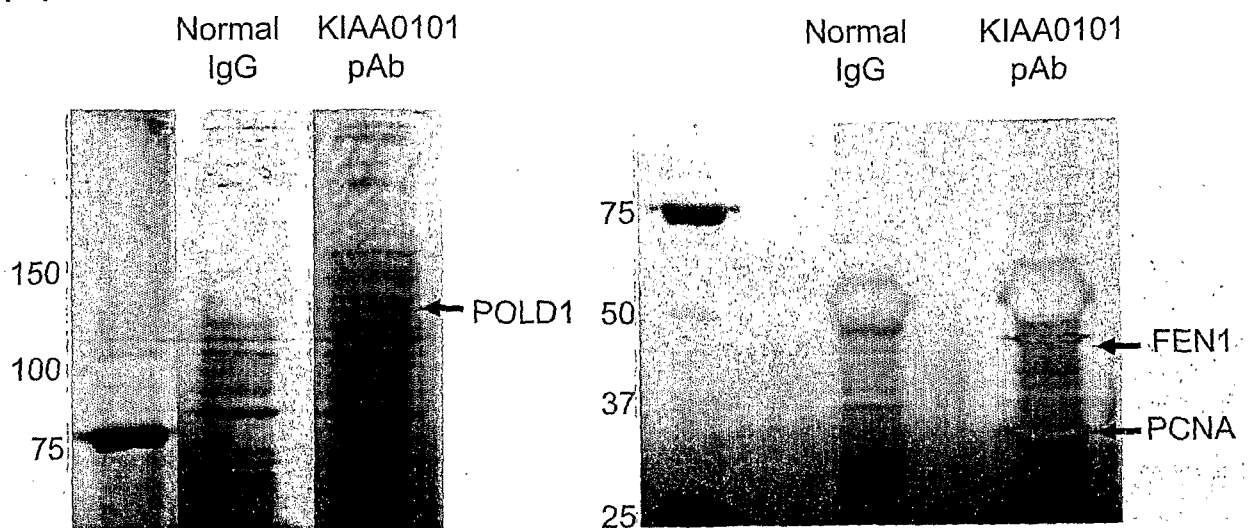
Fig.11



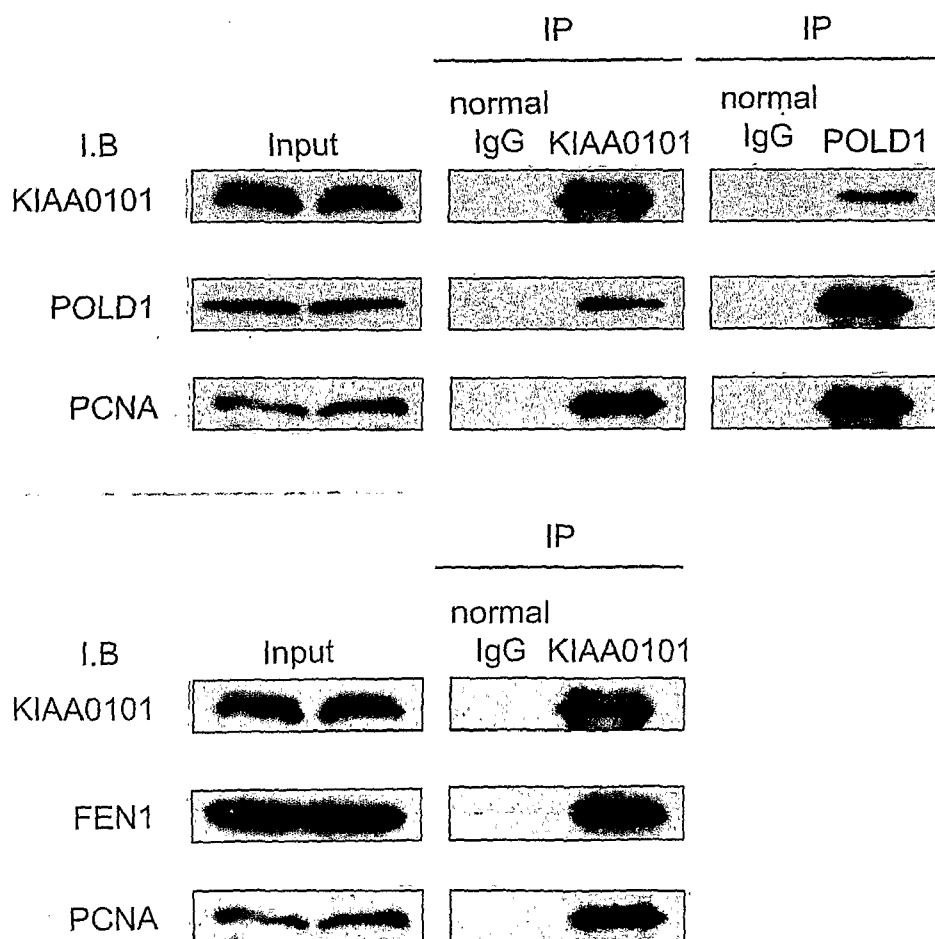
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Fig.12

(A)



(B)



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Fig.13

(A)

Scramble RRRRRRRRRRRRRGGG-IFKQWPRGETKPRVLSPKGF (SEQ ID NO:36)

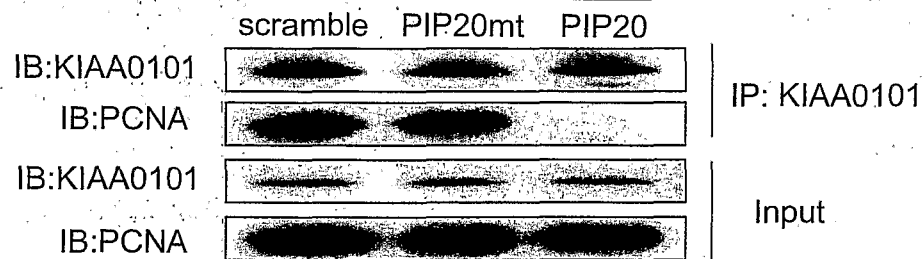
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PIP20 RRRRRRRRRRRRRGGG-VRPTPKWQKGIGEFFRLSPK (SEQ ID NO:34)

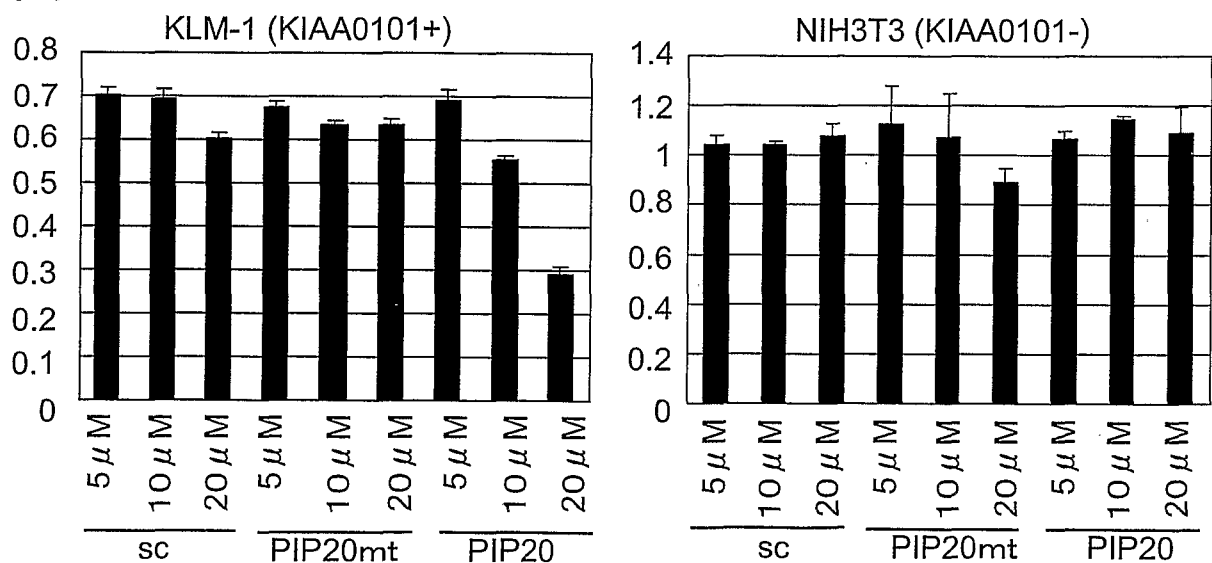
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PIP16 RRRRRRRRRRRRRGGG-TPKWQKGIGEFFRLSP (SEQ ID NO:38)

(B)



(C)



(D)

