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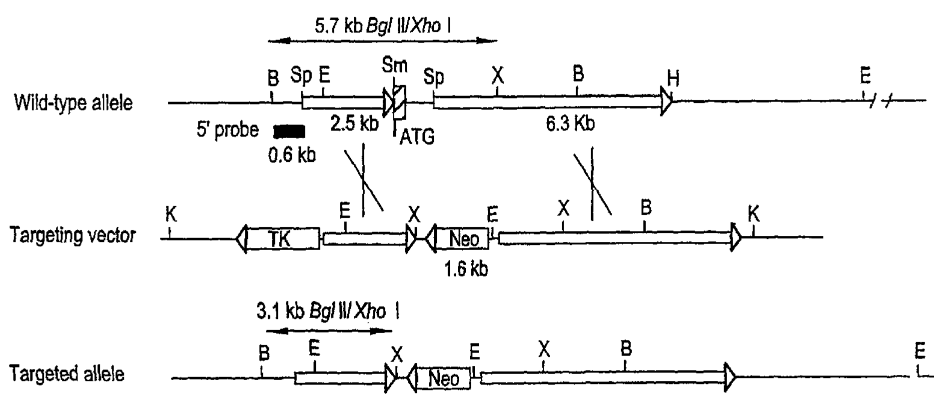
(54) Title: KINASE SUPPRESSOR OF RAS INACTIVATION FOR THERAPY OF RAS MEDIATED TUMORIGENESIS



(57) Abstract: The present invention relates to methods and compositions for the specific inhibition of kinase suppressor of Ras (KSR). In particular, the invention provides genetic approaches and nucleic acids for the specific inhibition of KSR, particularly of KSR expression. The invention relates to antisense oligonucleotides and the expression of nucleic acid which is substantially complementary to KSR RNA. Oligonucleotide and nucleic acid compositions are provided. The invention provides methods to inhibit KSR, including inhibition of KSR expression. Methods for blocking *gf* Ras mediated tumorigenesis, metastasis, and for cancer therapy are provided. Methods for conferring radiosensitivity to cells are also provided.

WO 2005/056756 A2

KINASE SUPPRESSOR OF RAS INACTIVATION FOR THERAPY OF RAS MEDIATED TUMORIGENESIS

GOVERNMENTAL SUPPORT

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FIELD OF THE INVENTION

[0002] The present invention relates to methods and compositions for the specific inhibition of of kinase suppressor of Ras (KSR). In particular, the invention provides genetic approaches and nucleic acids for the specific inhibition of KSR, particularly of KSR expression. The invention relates to antisense oligonucleotides and the expression of nucleic acid complementary to KSR RNA to specifically inhibit KSR and block *gf* Ras mediated tumorigenesis.

BACKGROUND OF THE INVENTION

[0003] Ras plays an essential role in oncogenic transformation and genesis. Oncogenic H-, K-, and N-Ras arise from point mutations limited to a small number of sites (amino acids 12, 13, 59 and 61). Unlike normal Ras, oncogenic *ras* proteins lack intrinsic

GTPase activity and hence remain constitutively activated (Trahey, M., and McCormick, F. (1987) Science 238: 542-5; Tabin, C. J. et al. (1982) Nature. 300: 143-9; Taparowsky, E. et al. (1982) Nature. 300: 762-5). The participation of oncogenic *ras* in human cancers is estimated to be 30% (Almoguera, C. et al (1988) Cell. *53*:549-54).

[0004] Mutations are frequently limited to only one of the *ras* genes, and the frequency is tissue- and tumor type-specific. K-*ras* is the most commonly mutated oncogene in human cancers, especially the codon-12 mutation. While oncogenic activation of H-, K-, and N-Ras arising from single nucleotide substitutions has been observed in 30% of human cancers (Bos, J.L. (1989) *Cancer Res* 49, 4682-9), over 90% of human pancreatic cancer manifest the codon 12 K-*ras* mutation (Almoguera, C. et al. (1988) *Cell* 53, 549-54; Smit, V.T. et al. (1988) *Nucleic Acids Res* 16, 7773-82; Bos, J.L. (1989) *Cancer Res* 49, 4682-9). Pancreatic ductal adenocarcinoma, the most common cancer of the pancreas, is notorious for its rapid onset and resistance to treatment. The high frequency of K-*ras* mutations in human pancreatic tumors suggests that constitutive Ras activation plays a critical role during pancreatic oncogenesis. Adenocarcinoma of the exocrine pancreas represents the fourth-leading cause of cancer-related mortality in Western countries. Treatment has had limited success and the five-year survival remains less than 5% with a mean survival of 4 months for patients with surgically unresectable tumors (Jemal, A et al (2002) *CA Cancer J Clin* 52, 23-47; Burris, H.A., 3rd et al. (1997) *J Clin Oncol* 15, 2403-13). This point mutation can be identified early in the course of the disease when normal cuboidal pancreatic ductal epithelium progresses to a flat hyperplastic lesion, and is considered causative in the pathogenesis of pancreatic cancer (Hruban, R.H. et al (2000) *Clin Cancer Res* 6, 2969-72; Tada, M. et al. (1996) *Gastroenterology* 110, 227-31). The regulation of oncogenic K-*ras* signaling in human pancreatic cancer, however, remains largely unknown.

[0005] K-*ras* mutations are present in 50% of the cancers of colon and lung (Bos, J. L. et al. (1987) Nature. 327: 293-7; Rodenhuis, S. et al. (1988) Cancer Res. 48: 5738-41). In cancers of the urinary tract and bladder, mutations are primarily in the H-*ras* gene (Fujita, J. et al. (1984) Nature. 309: 464-6; Visvanathan, K. V. et al. (1988) Oncogene Res. 3: 77-86). N-*ras* gene mutations are present in 30% of leukemia and liver cancer. Approximately 25% of skin lesions in humans involve mutations of the Ha-Ras (25% for squamous cell carcinoma and 28% for melanomas) (Bos, J. L. (1989) Cancer Res. *49*:4683-9; Migley, R. S. and Kerr, D. J. (2002) Crit Rev Oncol Hematol. *44*:109-20).50-60% of thyroid carcinomas are unique in having mutations in all three genes (Adjei, A. A. (2001) J Natl Cancer Inst. 93: 1062-74).

[0006] Constitutive activation of Ras can be achieved through oncogenic mutations or via hyperactivated growth factor receptors such as the EGFRs. Elevated expression and/or amplification of the members of the EGFR family, especially the EGFR and HER2, have been implicated in various forms of human malignancies (as reviewed in Prenzel, N. et al. (2001) Endocr Relat Cancer. 8: 11-31). In some of these cancers (including pancreas, colon, bladder, lung), EGFR/HER2 overexpression is compounded by the presence of oncogenic Ras mutations. Abnormal activation of these receptors in tumors can be attributed to overexpression, gene amplification, constitutive activation mutations or autocrine growth factor loops (Voldborg, B. R. et al. (1997) Ann Oncol. 8: 1197-206). For growth factor receptors, especially the EGFRs, amplification or /and overexpression of these receptors frequently occur in the cancers of the breast, ovary, stomach, esophagus, pancreatic, lung, colon neuroblastoma.

[0007] While various therapeutic strategies have been developed to inactivate key components of the Ras-Raf-MAPK cascade, specific inhibition of gain-of-function or constitutive Ras (*gf* Ras) action has not been achieved clinically (Adjei, A.A. (2001) *J*

Natl Cancer Inst 93, 1062-74; Cox, A.D. & Der, C.J. (2002) *Curr Opin Pharmacol* 2,388-93).

[0008] Therefore, in view of the aforementioned deficiencies attendant with prior art methods to inactivate or inhibit the Ras pathway, and particularly Ras-mediated cancers, it should be apparent that there still exists a need in the art for methods and compositions for specific inhibition of the Ras pathway and particularly for inhibition of *gf* Ras.

[0009] The citation of references herein shall not be construed as an admission that such is prior art to the present invention.

SUMMARY OF THE INVENTION

[0010] The present invention relates to methods and compositions for the specific inhibition of kinase suppressor of Ras (KSR). The compositions and methods of the present invention inhibit the expression and/or activity of KSR. In one aspect, the invention provides genetic approaches and nucleic acids for the specific inhibition of KSR. It is herein demonstrated that on specific inhibition of KSR the Ras pathway is disrupted and, specifically, Ras-mediated tumors and tumorigenesis is inhibited or blocked, existing tumors regress, metasasis is inhibited and proliferation of tumor or cancer cells is inhibited.

[0011] The present invention further relates to methods and means for radiosensitization, particularly of hyperproliferative, cancer or tumor cells, by specific inhibition of KSR expression and/or activity. In one such aspect, the hyperproliferative, cancer or tumor cells are resistant to ionizing radiation (IR) induced apoptosis or cell death. Inhibition of KSR, including by administration or expression of antisense oligonucleotide complementary to KSR, results in sensitization of cells to ionizing radiation, whereby the division or growth of cells is blocked or the cells are more effectively killed by IR than in the absence of the antisense oligonucleotide. The administration or expression of antisense oligonucleotide complementary to KSR facilitates and enhances cancer therapy, including radiation therapy.

[0012] The present invention further relates to methods and means for modulating angiogenesis, particularly by modulation of VEGF expression upon activation or inhibition of expression and/or activity of KSR. VEGF expression is modulated by specific inhibition or activation of KSR expression and/or activity. Activation or enhanced expression of KSR increases the amount or expression of VEGF in or by cells. Thus, activation or enhanced expression of KSR in a cell or tissue provides a method of stimulating angiogenesis. Inhibition, blockage or reduction of KSR decreases the amount or expression of VEGF, thereby having an anti-angiogenic effect. In one such aspect, VEGF expression of cancer or tumor cells is blocked by inhibition of KSR, including by administration or expression of antisense oligonucleotide complementary to KSR, resulting in inhibition of angiogenesis in the cancer or tumor. In one aspect, inhibition of KSR, including by administration or expression of antisense oligonucleotide complementary to KSR, provides a method of inhibiting angiogenesis in a tumor, tissue or cells expressing KSR, particularly a tissue or tumor expressing *gf* Ras or wherein the Ras pathway is hyperactivated or Ras is overexpressed or amplified.

[0013] The present invention provides oligonucleotides and nucleic acids which specifically inhibit or block the expression and activity of KSR. In particular, antisense oligonucleotides and the expression of nucleic acid complementary to KSR RNA specifically inhibits expression of KSR and blocks *gf* Ras mediated tumorigenesis. The present invention provides an oligonucleotide which is substantially complementary to a region of KSR RNA, wherein said oligonucleotide inhibits the expression of KSR. The invention further provides an oligonucleotide which is substantially complementary to a nucleic acid encoding mammalian KSR. In particular embodiments, oligonucleotides are provided which are substantially complementary to nucleic acid encoding mammalian KSR, particularly human and mouse KSR.

[0014] In one aspect, the invention provides a method of inhibiting the expression of mammalian KSR comprising contacting cells which express KSR with an effective amount of a nucleic acid which is complementary to a portion of the mRNA encoding KSR. In particular, the invention provides a method of inhibiting the expression of mammalian KSR, comprising contacting cells which express KSR with an effective amount of an antisense oligonucleotide, wherein the antisense oligonucleotide comprises a sequence specifically hybridizable to a portion of the coding region of human KSR (SEQ ID NO:24) selected from:

a. Nucleotides 1 to 18 (ATGGGAGAGAAGGAGGGC);
b. Nucleotides 205 to 222 (CTGGTCCGTTACATTTGT);
c. Nucleotides 247 to 264 (GTGGCTCCCGGTGAGAGG);
d. Nucleotides 298 to 315 (GACTGGCTGTACACTTTC);
e. Nucleotides 321 to 338 (GAGGCCGGAGGTGGTGCA);
f. Nucleotides 351 to 368 (AGATCCCCCGAGACCTCA);
g. Nucleotides 379 to 396 (ATGAATGAGGCCAAGGTG);
h. Nucleotides 511 to 528 (AGTTGGAGTTCATTGGAT); and
i. Nucleotides 531 to 548 (GCGGCGGGAAAGTGGCTC).

[0015] In another aspect, the invention provides a method of treating or preventing a hyperproliferative condition associated with the expression of *gf*-Ras or heightened expression of Ras in a mammal comprising administering to said mammal a therapeutically effective amount of an antisense oligonucleotide, wherein the antisense oligonucleotide comprises a sequence specifically hybridizable to a portion of the coding region of human KSR (SEQ ID NO:24) selected from:

a. Nucleotides 1 to 18 (ATGGGAGAGAAGGAGGGC);
b. Nucleotides 205 to 222 (CTGGTCCGTTACATTTGT);
c. Nucleotides 247 to 264 (GTGGCTCCCGGTGAGAGG);
d. Nucleotides 298 to 315 (GACTGGCTGTACACTTTC);
e. Nucleotides 321 to 338 (GAGGCCGGAGGTGGTGCA);
f. Nucleotides 351 to 368 (AGATCCCCCGAGACCTCA);
g. Nucleotides 379 to 396 (ATGAATGAGGCCAAGGTG);
h. Nucleotides 511 to 528 (AGTTGGAGTTCATTGGAT); and
i. Nucleotides 531 to 548 (GCGGCGGGAAAGTGGCTC).

[0016] In a further aspect, the invention provides a method of treating or inhibiting the progression of cancer in a mammal, comprising administering to a mammal a therapeutically effective amount of an antisense oligonucleotide, wherein the antisense oligonucleotide comprises a sequence specifically hybridizable to a portion of the coding region of human KSR (SEQ ID NO:24) selected from:

a. Nucleotides 1 to 18 (ATGGGAGAGAAGGAGGGC);
b. Nucleotides 205 to 222 (CTGGTCCGTTACATTTGT);
c. Nucleotides 247 to 264 (GTGGCTCCCGGTGAGAGG);
d. Nucleotides 298 to 315 (GACTGGCTGTACACTTTC);
e. Nucleotides 321 to 338 (GAGGCCGGAGGTGGTGCA);
f. Nucleotides 351 to 368 (AGATCCCCCGAGACCTCA);
g. Nucleotides 379 to 396 (ATGAATGAGGCCAAGGTG);
h. Nucleotides 511 to 528 (AGTTGGAGTTCATTGGAT); and
i. Nucleotides 531 to 548 (GCGGCGGGAAAGTGGCTC).

Cancers which are susceptible to the invention's method include cancer selected from the group of pancreatic cancer, lung cancer, skin cancer, urinary tract cancer, bladder cancer, liver cancer, thyroid cancer, colon cancer, intestinal cancer, leukemia, lymphoma, neuroblastoma, head and neck cancer, breast cancer, ovarian cancer, stomach cancer, esophageal cancer and prostate cancer.

[0017] The invention provides a method for sensitizing hyperproliferative, cancer or tumor cells in a mammal to ionizing radiation or other radiation or chemotherapy comprising administering to a mammal a therapeutically effective amount of one or more inhibitor of KSR expression and/or activity. In particular, the invention provides a method of conferring radiosensitivity to ionizing radiation in tumor cells in a mammal comprising administering to a mammal a therapeutically effective amount of a compound or agent which inhibits the expression of mammalian KSR protein.

[0018] In particular, such a method comprises administering to a mammal a therapeutically effective amount of an antisense oligonucleotide, wherein the antisense oligonucleotide is specifically hybridizable to a portion of the coding region of mRNA encoding human KSR (SEQ ID NO:24).

[0019] In a yet further aspect, the invention provides a method for modulating angiogenesis in a mammal by modulation of VEGF expression upon activation or inhibition of expression and/or activity of KSR. Inhibition, blockage or reduction of KSR decreases the amount or expression of VEGF, thereby having an anti-angiogenic effect. In a particular such method, VEGF expression of cancer or tumor cells is blocked by inhibition of KSR, including by administration or expression of antisense oligonucleotide complementary to KSR, resulting in inhibition of angiogenesis in the cancer or tumor. In particular, the invention provides a method of inhibiting angiogenesis of tumor cells in a mammal comprising administering to a mammal a therapeutically effective amount of a compound or agent which inhibits the expression of mammalian KSR protein.

[0020] In particular, such a method comprises administering to a mammal a therapeutically effective amount of an antisense oligonucleotide, wherein the antisense oligonucleotide is specifically hybridizable to a portion of the coding region of mRNA encoding human KSR (SEQ ID NO:24).

[0021] The oligonucleotides used in the method of the present invention may be labeled with a detectable label. In particular aspects, the label may be selected from enzymes, ligands, chemicals which fluoresce and radioactive elements. In the instance where a radioactive label, such as the isotopes ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{36}Cl, ^{51}Cr, ^{57}Co, ^{58}Co, ^{59}Fe, ^{90}Y, ^{125}I, ^{131}I, and ^{186}Re are used, known currently available counting procedures may be utilized.

[0022] In the instance where the label is an enzyme, detection may be accomplished by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques known in the art.

[0023] In a particular aspect, the oligonucleotides may be modified, either by manipulation of the chemical backbone of the nucleic acids or by covalent or non-covalent attachment of other moieties. In each or any case, such manipulation or attachment may serve to modify the stability, cellular, tissue or organ uptake, or otherwise enhance efficacy of the oligonucleotides.

[0024] In further aspects of the invention, the oligonucleotides may be covalently linked to other molecules, including but not limited to polypeptides, carbohydrates, lipid or lipid-like moieties, ligands, chemical agents or compounds, which may serve to enhance the uptake, stability or to target the oligonucleotides.

[0025] In further embodiments, the oligonucleotides are modified in their chemical backbone. In a particular embodiment, the oligonucleotides comprise at least one phosphorothioate (P-S) linkage.

[0026] Recombinant DNA molecules comprising a nucleic acid sequence which encodes on transcription an antisense RNA complementary to mammalian KSR RNA or a portion thereof are provided by the invention. Further, the recombinant DNA molecules comprise a nucleic acid sequence wherein said nucleic acid sequence is operatively linked to a transcription control sequence.

[0027] Cell lines transfected with these recombinant DNA molecules are also included in the invention.

2004297594 16 Sep 2009

[0028] In a further aspect, an expression vector is provided which is capable of expressing a nucleic acid which is substantially complementary to the coding sequence of KSR RNA, or a portion thereof, wherein said nucleic acid inhibits the expression of KSR.

[0029] In a particular aspect, this includes an expression vector capable of expressing an oligonucleotide which is substantially complementary to the CA1 region of the coding sequence of KSR RNA, particularly of mouse or human KSR (SEQ ID NO: 1 or SEQ ID NO: 25), or a portion thereof, wherein said oligonucleotide inhibits the expression of KSR.

[0030] Compositions of the oligonucleotides used in the above methods are an additional aspect of the invention. The invention includes a composition comprising an oligonucleotide which is substantially complementary to a region of KSR RNA and a pharmaceutically acceptable carrier or diluent. The invention thus provides a pharmaceutical composition comprising a therapeutically effective amount of an oligonucleotide which is substantially complementary to a region of KSR RNA and a pharmaceutically acceptable carrier or diluent.

[0031] In a further aspect, compositions are provided comprising one or more chemotherapeutic or radiotherapeutic agent and an oligonucleotide which is targeted to a mRNA encoding mammalian KSR and which inhibits KSR expression.

[0032] In an additional embodiment, the invention provides a composition comprising an expression vector and a pharmaceutically acceptable carrier or diluent, wherein said expression vector is capable of expressing nucleic acid which is substantially complementary to the coding sequence of KSR RNA, or a portion thereof, wherein said nucleic acid inhibits the expression of KSR.

[0033] The present invention naturally contemplates several means for preparation of the agents and antisense oligonucleotides, including as illustrated herein known recombinant techniques, and the invention is accordingly intended to cover such synthetic preparations within its scope. The knowledge of the cDNA and amino acid sequences of KSR as disclosed herein facilitates the preparation of the nucleic acids of the invention by such recombinant techniques, and accordingly, the invention extends to expression vectors prepared from the disclosed DNA sequences for expression in host systems by recombinant DNA techniques, and to the resulting transformed hosts.

[0034] Other objects and advantages will become apparent to those skilled in the art from a review of the following description which proceeds with reference to the following illustrative drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

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2004297594 16 Sep 2009

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[0035] FIGURE 1 depicts targeted disruption of the *ksr* gene in mice. **A**, Strategy for targeting the *ksr* allele. Simplified restriction maps of the 5' region of the wild-type *ksr* allele, the targeting vector, and the mutated allele are shown. Homologous recombination with endogenous *ksr* replaces an internal 1.1-kb *Sma*I-*Spe*I genomic fragment with a Neo cassette. **B**, Southern blot analysis of an ES clone showing the correct insertion of the targeting construct. Genomic DNA isolated from ES cells was digested with *Bgl*II and *Xho*I and hybridized to the 5' probe located just outside the 5'arm of the *ksr* targeting region as shown in **A**. The wild-type allele yields a 5.7-kb fragment whereas the mutant allele yields a 3.1-kb fragment. **C**, Genotyping of $ksr^{-/-}$ mice by PCR. The size of the PCR product is 493 bp for the wt allele and 312 bp for the mutated allele. **D**, Expression of *ksr* in wild type mouse embryos. The sizes of the two transcripts are 6.4 kb and 7.4 kb. **E**, Northern blot analysis of tissue *ksr* mRNAs. Poly-A^+ RNA, isolated from different tissues of adult $ksr^{+/+}$, $ksr^{+/-}$, and $ksr^{-/-}$ mice, was hybridized with a probe corresponding to domains CA2-CA4 in *ksr* cDNA. mRNA from NIH3T3 cells was used as control. **F**, KSR protein expression. Lysates prepared from wild-type and $ksr^{-/-}$ tissues were analyzed by western blot with a specific anti-KSR monoclonal antibody. Note that brain expresses the slightly shorter B-KSR1 isoform while lung and spleen express the longer KSR1 isoform. Lysates were also prepared from two independent sets of $ksr^{+/+}$ and $ksr^{-/-}$ MEFs. Equal loading was confirmed by reprobing blots with an anti-α-tubulin antibody.

[0036] FIGURE 2 depicts skin phenotype in newborn $ksr^{-/-}$ mice. Full thickness skin cuts of 10-day old $ksr^{+/+}$, $ksr^{-/-}$ and $egfr^{-/-}$ mice were sectioned 4-6 μm thick, placed on glass slides, and stained with hematoxylin and eosin. s –serpentine, bl –blister, do-disoriented.

[0037] FIGURE 3 depicts defects in EGF- and TPA- induced MAPK signaling and proliferation in $ksr^{-/-}$ MEFs. **A**, Western blot analysis of MAPK activity upon EGF and TPA treatments. Low-passage MEFs derived from $ksr^{+/+}$ and $ksr^{-/-}$ were made quiescent by 48 h incubation in serum-free medium and stimulated with low doses of EGF for 3 min(upper panel) or with TPA for 10 min (lower panel). Cells were lysed in NP40 buffer and activation of the MAPK cascade was examined by western blot with anti-phospho specific antibodies for the activated forms of MAPK(ERK1/2). Shown are representative blots from one of four independent experiments. **B**, Activation of endogenous Raf-1 upon EGF (upper panel) and TPA (lower panel) treatments were determined by Raf-1 activity assay as described herein in Methods. MEK1 phosphorylation was examined by western blot with anti-phospho specific antibodies for the activated forms of MEK1. Shown are representative blots from one or four independent experiments. **C**, Proliferation of MEFs. 0.15 x 10^6 $ksr^{+/+}$ or $ksr^{-/-}$ low-passage MEFs were seeded on 60 mm plates and grown as described in Methods. Cells were trypsinized every other day and counted by hemacytometer. Data (mean±SD) are compiled from three independent experiments.

[0038] FIGURE 4. Disruption of *ksr* gene abrogated oncogenic Ras-mediated tumorigenesis in $ksr^{-/-}$ mice. **A,** RT-PCR detection of *v*-Ha-*ras* expression from total RNA isolated from the epidermis of Tg.AC/$ksr^{+/+}$ and Tg.AC/$ksr^{-/-}$ mice following TPA treatment. Intron spanning primers specific for the 3'UTR region of the *v*-Ha-*ras* transgene were used. The larger 279 bp amplicon, detected in the absence of reverse transcriptase [RT(-)], is derived from DNA and unspliced RNA. The smaller 214 bp amplicon is derived from spliced mRNA and is indicative of transgene expression. **B,** Mice, grouped according to genotype (10/group), were treated with 5μg of TPA twice a week for 15 weeks. Papillomas were counted weekly for 20 weeks.

[0039] FIGURE 5. Inducible A431-Tet-Off-pTRE–KSR cells. **A** and **B**, Western blot analysis of wild type Flag-KSR-S and Flag-DN-KSR expression *(***A**), and inhibition of endogenous KSR1 expression by KSR-AS *(***B**). Flag-KSR-S and DN-KSR were immunoprecipitated (IP) with the monoclonal anti-Flag (M2) antibody and detected by WB. The identity of Flag-KSR was confirmed by re-probing with a monoclonal anti-KSR antibody (BD Biosciences). Endogenous KSR1 was immunoprecipitated as described in Methods and detected as above. **C**, Dose-dependent inhibition of Flag-KSR-S expression by doxycycline. KSR-S cells were treated with indicated doses of Dox for 24 h and Flag-KSR-S expression after Dox treatment was determined by WB as above. **D** and **E**, Inactivation of KSR1 by KSR-AS or DN-KSR leads to alterations in morphology *(***D***)*, and the development of a multinuclei phenotype *(***E***)*. A431-pTRE cells were examined under the phase-contrast microscope and photographed at 20x magnification for cell morphology *(***D***)* and 40x magnification for multinucleation *(***E***)*.

[0040] FIGURE 6. Inactivation of KSR1 abolishes EGF-stimulated biological responses in A431 cells. **A**, Cell proliferation assay without ***(i)*** and with ***(ii)*** EGF stimulation. Proliferation assays were performed as described in Methods. **B**, Cell cycle distribution of A431-pTRE cells was determined by FACS analysis as described in Methods. The proportion of cells in the different phases of the cell cycle was calculated from the experimental fluorescence histograms. **C**, Matrigel invasion assay in response to EGF stimulation. To optimize the stimulatory effect of overexpression of KSR-S on A431 cell invasion, the assay was terminated after 12 h ***(i)***. To maximize the inhibitory effect of KSR-AS and DN-KSR on A431 cell invasion, the assay was terminated after 18 h ***(ii)***. **D**, Soft agar colony formation assays in response to EGF stimulation were performed as in Methods. For each cell line or treatment, 4 plates were counted. These results represent one of four similar studies.

[0041] FIGURE 7. Inactivation of KSR1 prevents A431 tumorigenesis. **A**, Growth curve of A431 tumors. 10^6 A431-pTRE cells were injected s.c. into nude mice as described in Methods. To determine the specificity of KSR-S on A431 tumorigenesis, Dox (100 mg/ml) was added to the drinking water of a group of KSR-S tumor-bearing mice (KSR-S + Dox) 3 days prior to tumor implantation and continued throughout the experiment to turn off KSR-S expression. Mice receiving KSR-AS and DN-KSR cells were monitored up to 120 days. These results represent one of three similar experiments. There were 5 mice in each experimental group. **B**, H&E staining of A431 tumors. Formalin-fixed, paraffin-embedded and 5 mm-cut A431-pTRE tumor sections were stained with H&E as described in Methods. Black arrows in ***(i)*** and ***(ii)*** indicate squamous differentiation. Black arrows in ***(iii)*** and ***(iv)*** indicate multinucleated tumor cells, and ***(v)*** is the enlargement of the framed field in ***(iii)*** of a multinucleated cell.

[0042] FIGURE 8. Inactivation of KSR1 by AS-ODN attenuates A431 tumorigenesis. **A**, Immunofluorescence staining of endogenous KSR1 expression after treatment with 1 mM Control- or AS-ODNs was performed as in Methods. Nuclei were counter stained with DAPI. To compare the intensity of fluorescence labeling, all images of KSR expression were taken with the same exposure time. **B** and **C**, Dose-dependent inhibition of A431 cell proliferation ***(B)*** and invasion ***(C)*** by AS-ODN treatment. For the proliferation assay, 30% confluent A431 cells were treated with the indicated doses of Control- or AS-ODNs as in Fig. 3. Cell proliferation after ODN treatment was calculated as percent of non-treated controls. Invasion assays were set up after 48 h of ODN treatment as above. **D**, Attenuation of A431 tumorigenesis by continuous infusion of AS-ODN at 5 mg/kg/day. A431 seed tumor fragments freshly prepared as described in Methods, were transplanted s.c. into the right lateral flank of nude mice. Continuous infusion of ODNs was initiated 2 days prior to tumor transplantation. There were 5 mice in each treatment group. These results represent one of three similar experiments.

[0043] FIGURE 9. Inactivation of KSR1 by AS-ODN inhibits oncogenic K-*ras* signaling *in vitro* in PANC-1. **A**, Dose-dependent inhibition of PANC-1 cell proliferation by AS-ODN treatment. PANC-1 cells were treated with the indicated doses of Control- or AS-ODNs and cell proliferation assays were performed as in FIGURE 7. **B**, AS-ODN treatment (5 μM) attenuated the proliferation of a panel of human pancreatic cancer cell lines. The seeding density for each cell lines was determined in preliminary studies so that all cell lines were 30-40% confluent when transfected with ODNs. **C** and **D**, c-Raf-1 is epistatic to KSR1. PANC-1 cells were first treated with Sense- or AS-ODNs for 48 h and then transfected with the BXB-Raf as in Methods. 48 h after transfection, invasion and colony formation assays were set up as in FIGURE 7. The inhibitory effect of AS-ODN on PANC-1 cell invasion ***(C)*** and transformation ***(D)*** was reversed by dominant positive BXB-Raf. **E**, AS-ODN treatment inhibited endogenous KSR1 expression in PANC-1 cells. Endogenous KSR1 was immunoprecipitated from non-treated (NT), Sense-ODN-treated or AS-ODN-treated PANC-1 cells, and KSR1 expression was determined by WB as described in Methods. Purified Flag-KSR served as a positive control for the WB. **F**, MAPK and PI-3 kinase activation in AS-ODN-treated and BXB-Raf-1-transfected PANC-1 cells in response to EGF were determined by WB analysis using phospho-MAPK and phospho-Akt specific antibodies as described in Methods. Under these conditions, b-actin and total Akt were unchanged (not shown). These results represent one of three similar experiments.

[0044] FIGURE 10. AS-ODN treatment abolished PANC-1 and A549 tumorigenesis *in vivo*. **A**, Continuous infusion of AS-ODN abolished PANC-1 tumor growth. PANC-1 xenografts derived either from 10^6 PANC-1 cells **(i)**, or from freshly harvested seed PANC-1 tumors **(ii)** were transplanted into nude mice as described in Methods. **A (i),** established PANC tumors (approximately 100 mm^3) were treated with 10 mg/kg/day of Control- or AS-ODNs for 14 days. Mice bearing regressed AS-ODN-treated tumors were monitored up to 4 weeks. **A(ii),** freshly prepared PANC-1 seed tumor fragments were transplanted into nude mice as above. Infusion with ODNs was initiated two days prior to tumor implantation and continued for an additional 14 days. These results represent one of three similar experiments. There were 5 mice for each treatment group. **B,** AS-ODN treatment inhibited endogenous tumoral KSR1 expression. Tumoral KSR1 was immunoprecipitated from Saline-, Sense- or AS-ODN-treated PANC-1 tumors and its expression determined by WB as above. **C**, Inhibition of *ksr*1 had no effect of Ras activation in PANC-1 tumors. Ras activation status, measured by the amount of GTP-Ras in Saline-, Control-ODN-, Sense-ODN- or AS-ODN-treated PANC-1 tumors was determined using the Ras activation assay kit. **D**, KSR AS-ODN treatment prevented A549 tumor growth **(i)** and inhibited lung metastases via systemic dissemination **(ii).** A549 seed tumor fragments, freshly prepared as in Methods, were transplanted to nude mice. Treatment with control-or AS-ODNs were initiated when tumors reached 150 mm^3 and continued for additional 18 days **(i).** When animals were sacrificed at the end of the experiment, lungs were resected from control- or AS-ODN-treated mice and stained with Indian ink to visualize surface lung metastases derived via systemic dissemination **(ii)**. These results represent one of three similar experiments. There were 5 mice for each treatment group.

[0045] FIGURE 11 depicts a comparative alignment of the mouse KSR polypeptide sequence (SEQ ID NO: 9) and human KSR polypeptide sequence (SEQ ID NO: 10).

[0046] FIGURE 12. **A**, depicts the nucleic acid coding (cDNA) sequence of mouse *ksr* (SEQ ID NO: 11). **B**, depicts the partial nucleic acid coding (cDNA) sequence of human *ksr* (SEQ ID NO:12).

[0047] FIGURE 13 depicts the specific and dose-dependent inhibition of PANC-1 cell proliferation by AS-ODN treatment. **A**, Dose-dependent inhibition of PANC-1 cell proliferation by AS-ODN treatment; proliferation of K562 cells is not inhibited by AS-ODN treatment. **B**, Western blot analysis of endogenous KSR1 gene expression in K562 and PANC-1 cells. Treatment of K562 cells with 5μM KSR AS-ODN-1 elicited comparable reduction of endogenous KSR1 gene expression (over 80%) to that observed in PANC-1 cells.

[0048] **FIGURE 14** depicts the human KSR1 full length mRNA sequence (SEQ ID NO:24).

[0049] **FIGURE 15** depicts the human KSR nucleic acid and protein sequence annotated with the locations of the CA1 to CA5 domains and the target sequences for AS-ODNs. The full length human KSR1 protein is predicted to have 866 amino acids.

[0050] **FIGURE 16** depicts the mouse KSR nucleic acid and protein sequence annotated with the locations of the CA1 to CA5 domains and the target sequences for the AS-ODNs.

[0051] **FIGURE 17** depicts the human KSR-1 full length mRNA sequence with the nucleic acid target sequences for human AS-ODN1 through AS-ODN12 depicted.

[0052] **FIGURE 18** depicts proliferation assay of PANC-1 cells treated with control ODN, AS-ODN2(181-198) (mouse) (AS-ODN2-old) and AS-ODN2(154-171) (human) (AS-ODN2-new).

[0053] **FIGURE 19** presents percentage of ionizing-radiation induced apoptosis as scored with Annexin V staining in A431 cells expressing vector, wild-type Flag-KSR (KSR-S), Flag-AS-KSR (KSR-AS) or dominant-negative Flag-Ki-KSR (Ki-KSR).

[0054] **FIGURE 20** presents percentage of ionizing-radiation induced apoptosis as scored with Annexin V staining in A431 cells treated with AS-ODN2(214-231), control ODN or expressing vector.

[0055] **FIGURE 21A and 21B** depicts expression of KSR-AS radiosensitizes A431 cells to ionizing radiation.

[0056] **FIGURE 22A and 22B** depicts inactivation of KSR (by KSR-AS expression) abrogates oth baseline and EGF-stimulated VEGF production in A431 cells.

DETAILED DESCRIPTION

[0057] In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook et al, "Molecular Cloning: A Laboratory Manual" (1989); "Current Protocols in Molecular Biology" Volumes I-III [Ausubel, R. M., ed. (1994)]; "Cell Biology: A Laboratory Handbook" Volumes I-III [J. E. Celis, ed. (1994))]; "Current Protocols in Immunology" Volumes I-III [Coligan, J. E., ed. (1994)]; "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" [B.D. Hames & S.J. Higgins eds. (1985)]; "Transcription And Translation" [B.D. Hames & S.J. Higgins, eds. (1984)]; "Animal Cell Culture" [R.I. Freshney, ed. (1986)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

[0058] Therefore, if appearing herein, the following terms shall have the definitions set out below.

[0059] The terms “oligonucleotides”, “antisense”, “antisense oligonucleotides”, “KSR ODN”, “KSR antisense” and any variants not specifically listed, may be used herein interchangeably, and as used throughout the present application and claims refer to nucleic acid material including single or multiplenucleic acids, and extends to those oligonucleotides complementary to the nucleic acid sequences described herein, including as presented in FIGURE 12 A, 12 B and 14 and in SEQ ID NOS: 11, 12, and 24, including conserved and activity domains thereof as depicted in FIGURES 11, 15 and

16, and having the profile of activities set forth herein and in the Claims, particularly in being capable of inhibiting the expression of KSR. In particular, the oligonucleotides of the present invention may be substantially complementary to nucleic acid sequence specific to KSR, as provided in SEQ ID NO: 1 or SEQ ID NO:25, or to a portion thereof, as provided for example in SEQ ID NO: 3, 4, 5 and 27. Exemplary oligonucleotides include any of SEQ ID NOS 6-8 and SEQ ID NOS: 28-39. Accordingly, nucleic acids or analogs thereof displaying substantially equivalent or altered activity are likewise contemplated. These modifications may be deliberate, for example, such as modifications obtained through site-directed mutagenesis, or may be accidental, such as those obtained through mutations in hosts that are producers of the nucleic acids or of KSR.

[0060] NH_2 refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature, *J. Biol. Chem.*, **243**:3552-59 (1969), abbreviations for amino acid residues are shown in the following Table of Correspondence:

TABLE OF CORRESPONDENCE

SYMBOL		AMINO ACID
1-Letter	3-Letter	
Y	Tyr	tyrosine
G	Gly	glycine
F	Phe	phenylalanine
M	Met	methionine
A	Ala	alanine

S	Ser	serine
I	Ile	isoleucine
L	Leu	leucine
T	Thr	threonine
V	Val	valine
P	Pro	proline
K	Lys	lysine
H	His	histidine
Q	Gln	glutamine
E	Glu	glutamic acid
W	Trp	tryptophan
R	Arg	arginine
D	Asp	aspartic acid
N	Asn	asparagine
C	Cys	cysteine

[0061] It should be noted that all amino-acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino-acid residues. The above Table is presented to correlate the three-letter and one-letter notations which may appear alternately herein.

[0062] A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*; i.e., capable of replication under its own control.

[0063] A "vector" is a replicon, such as plasmid, phage, virus, retrovirus or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

[0064] A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

[0065] An "origin of replication" refers to those DNA sequences that participate in DNA synthesis.

[0066] A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

[0067] Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

[0068] A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

[0069] An "expression control sequence" is a DNA sequence that controls and regulates the transcription and translation of another DNA sequence. A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

[0070] A "signal sequence" can be included before the coding sequence. This sequence encodes a signal peptide, N-terminal to the polypeptide, that communicates to the host cell to direct the polypeptide to the cell surface or secrete the polypeptide into the media, and this signal peptide is clipped off by the host cell before the protein leaves the cell. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes.

[0071] The term "oligonucleotide," as used herein in referring to a nucleic acid of the present invention, is defined as a molecule comprised of two or more ribonucleotides, preferably more than three. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide. In particular, and in accordance with the present invention, the oligonucleotide should particularly associate with the RNA encoding KSR and should be of the appropriate sequence and size or length so as to specifically and stably associate with the target RNA such that expression (i.e., translation) of the RNA is blocked or such that stability of the RNA is negatively affected. In one particular aspect of the invention, the antisense oligonucleotides of the present invention are from about 8 to about 50 nucleotides in length, particularly oligonucleotides from 10 to 30 nucleotides in length, particularly oligonucleotides from 15 to 25 nucleotides.

[0072] The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand, is induced, i.e., in the presence of nucleotides and an inducing agent such as a DNA polymerase and at a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired extension product in the presence of the inducing agent. The exact length of the primer will depend upon many factors, including temperature, source of primer and use of the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides.

[0073] The primers herein are selected to be "substantially" complementary to different strands of a particular target DNA sequence. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to hybridize therewith and thereby form the template for the synthesis of the extension product.

[0074] As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

[0075] A cell has been "transformed" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

[0076] Two DNA sequences are "substantially homologous" when at least about 75% (preferably at least about 80%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Maniatis et al., *supra*; DNA Cloning, Vols. I & II, *supra*; Nucleic Acid Hybridization, *supra*.

[0077] It should be appreciated that also within the scope of the present invention are DNA or nucleic acid sequences which code for KSR having the same amino acid sequence as the KSR sequences disclosed herein, including SEQ ID NO: 11 and SEQ ID NO: 12, but which are degenerate these sequences. By "degenerate to" is meant that a different three-letter codon is used to specify a particular amino acid. It is well known in the art that the following codons can be used interchangeably to code for each specific amino acid:

[0078] Phenylalanine (Phe or F)	UUU or UUC
Leucine (Leu or L)	UUA or UUG or CUU or CUC or CUA or CUG
Isoleucine (Ile or I)	AUU or AUC or AUA
Methionine (Met or M)	AUG
Valine (Val or V)	GUU or GUC of GUA or GUG
Serine (Ser or S)	UCU or UCC or UCA or UCG or AGU or AGC
Proline (Pro or P)	CCU or CCC or CCA or CCG
Threonine (Thr or T)	ACU or ACC or ACA or ACG
Alanine (Ala or A)	GCU or GCG or GCA or GCG
Tyrosine (Tyr or Y)	UAU or UAC
Histidine (His or H)	CAU or CAC

Glutamine (Gln or Q)	CAA or CAG
Asparagine (Asn or N)	AAU or AAC
Lysine (Lys or K)	AAA or AAG
Aspartic Acid (Asp or D)	GAU or GAC
Glutamic Acid (Glu or E)	GAA or GAG
Cysteine (Cys or C)	UGU or UGC
Arginine (Arg or R)	CGU or CGC or CGA or CGG or AGA or AGG
Glycine (Gly or G)	GGU or GGC or GGA or GGG
Tryptophan (Trp or W)	UGG
Termination codon	UAA (ochre) or UAG (amber) or UGA (opal)

[0079] It should be understood that the codons specified above are for RNA sequences. The corresponding codons for DNA have a T substituted for U.

[0080] Mutations can be made in *ksr* such that a particular codon is changed to a codon which codes for a different amino acid. Such a mutation is generally made by making the fewest nucleotide changes possible. A substitution mutation of this sort can be made to change an amino acid in the resulting protein in a non-conservative manner (i.e., by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to another grouping) or in a conservative manner (i.e., by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to the same grouping). Such a conservative change generally leads to less change in the structure and function of the resulting protein. A non-conservative change is more likely to alter the structure, activity or function of the resulting protein. The present invention should be considered to include sequences containing conservative changes which do not significantly alter the activity or binding characteristics of the resulting protein.

[0081] The following is one example of various groupings of amino acids:

Amino acids with nonpolar R groups

Alanine, Valine, Leucine, Isoleucine, Proline, Phenylalanine, Tryptophan, Methionine

[0082] Amino acids with uncharged polar R groups

Glycine, Serine, Threonine, Cysteine, Tyrosine, Asparagine, Glutamine

[0083] Amino acids with charged polar R groups (negatively charged at Ph 6.0)

Aspartic acid, Glutamic acid

[0084] Basic amino acids (positively charged at pH 6.0)

Lysine, Arginine, Histidine (at pH 6.0)

[0085] Another grouping may be those amino acids with phenyl groups:

Phenylalanine, Tryptophan, Tyrosine

[0086] Another grouping may be according to molecular weight (i.e., size of R groups):

Glycine	75
Alanine	89
Serine	105
Proline	115
Valine	117
Threonine	119
Cysteine	121
Leucine	131
Isoleucine	131
Asparagine	132

Aspartic acid	133
Glutamine	146
Lysine	146
Glutamic acid	147
Methionine	149
Histidine (at pH 6.0)	155
Phenylalanine	165
Arginine	174
Tyrosine	181
Tryptophan	204

[0087] Particularly preferred substitutions are:

- Lys for Arg and vice versa such that a positive charge may be maintained;
- Glu for Asp and vice versa such that a negative charge may be maintained;
- Ser for Thr such that a free -OH can be maintained; and
- Gln for Asn such that a free NH_2 can be maintained.

[0088] Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced a potential site for disulfide bridges with another Cys. A His may be introduced as a particularly "catalytic" site (i.e., His can act as an acid or base and is the most common amino acid in biochemical catalysis). Pro may be introduced because of its particularly planar structure, which induces β-turns in the protein's structure.

[0089] Two amino acid sequences are "substantially homologous" when at least about 70% of the amino acid residues (preferably at least about 80%, and most preferably at least about 90 or 95%) are identical, or represent conservative substitutions.

[0090] A "heterologous" region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

[0091] The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human.

[0092] The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to prevent, and preferably reduce by at least about 30 percent, more preferably by at least 50 percent, more preferably by at least 70 percent, most preferably by at least 90 percent, a clinically significant change in the mitotic activity of a target cellular mass, or other feature of pathology such as for example, reduced tumor mass, reduced tumor cell proliferation, reduction in metastatic capacity, or enhanced apoptosis, as may attend its presence and activity.

[0093] As used herein, "pg" means picogram, "ng" means nanogram, "ug" or "μg" mean microgram, "mg" means milligram, "ul" or "μl" mean microliter, "ml" means milliliter, "l" means liter.

[0094] A DNA sequence is "operatively linked" to an expression control sequence when the expression control sequence controls and regulates the transcription and translation of that DNA sequence. The term "operatively linked" includes having an appropriate start signal (e.g., ATG) in front of the DNA sequence to be expressed and maintaining the correct reading frame to permit expression of the DNA sequence under the control of the expression control sequence and production of the desired product encoded by the DNA sequence. If a gene that one desires to insert into a recombinant DNA molecule does not contain an appropriate start signal, such a start signal can be inserted in front of the gene.

[0095] The term "standard hybridization conditions" refers to salt and temperature conditions substantially equivalent to 5 x SSC and 65°C for both hybridization and wash. However, one skilled in the art will appreciate that such "standard hybridization conditions" are dependent on particular conditions including the concentration of sodium and magnesium in the buffer, nucleotide sequence length and concentration, percent mismatch, percent formamide, and the like. Also important in the determination of "standard hybridization conditions" is whether the two sequences hybridizing are RNA-RNA, DNA-DNA or RNA-DNA. Such standard hybridization conditions are easily determined by one skilled in the art according to well known formulae, wherein hybridization is typically 10-20°C below the predicted or determined T_m with washes of higher stringency, if desired.

[0096] The present invention relates to methods and compositions for the specific inhibition of expression and/or activity of kinase suppressor of Ras (KSR). In particular, the invention provides genetic approaches and nucleic acids for the specific inhibition of KSR. It is herein demonstrated that on specific inhibition of KSR the Ras pathway is disrupted and, specifically, that Ras-mediated tumors, tumorigenesis and metastasis regress, are inhibited, or are blocked. In particular antisense oligonucleotides and the

2004297594 16 Sep 2009

expression of nucleic acid complementary to KSR RNA specifically inhibits expression of KSR and blocks *gf* Ras mediated tumorigenesis.

[0097] The present invention provides an oligonucleotide which is substantially complementary to a region of KSR RNA, wherein said oligonucleotide inhibits the expression of KSR. The invention further provides an oligonucleotide which is substantially complementary to a nucleic acid encoding mammalian KSR. In a particular embodiment, an oligonucleotide is provided which is substantially complementary to a nucleic acid encoding human KSR.

[0098] Methods for inhibiting expression of KSR are provided. In one aspect, a method of inhibiting the expression of mammalian KSR comprising contacting cells which express KSR with an effective amount of a nucleic acid which is complementary to a portion of the mRNA encoding KSR is provided. In particular, a method of inhibiting the expression of mammalian KSR is provided, comprising contacting cells which express KSR with an effective amount of an antisense oligonucleotide, wherein the antisense oligonucleotide comprises a sequence specifically hybridizable to a portion of the coding region of human KSR (SEQ ID NO:24) selected from:

a. Nucleotides 1 to 18 (ATGGGAGAGAAGGAGGGC);
b. Nucleotides 205 to 222 (CTGGTCCGTTACATTTGT);
c. Nucleotides 247 to 264 (GTGGCTCCCGGTGAGAGG);
d. Nucleotides 298 to 315 (GACTGGCTGTACACTTTC);
e. Nucleotides 321 to 338 (GAGGCCGGAGGTGGTGCA);
f. Nucleotides 351 to 368 (AGATCCCCCGAGACCTCA);
g. Nucleotides 379 to 396 (ATGAATGAGGCCAAGGTG);
h. Nucleotides 511 to 528 (AGTTGGAGTTCATTGGAT); and
i. Nucleotides 531 to 548 (GCGGCGGGAAAGTGGCTC).

[0099] In another aspect, the invention provides a method of treating or preventing a hyperproliferative condition associated with the expression of *gf*-Ras or heightened expression of Ras in a mammal comprising administering to said mammal a therapeutically effective amount of an antisense oligonucleotide, wherein the antisense oligonucleotide comprises a sequence specifically hybridizable to a portion of the coding region of human KSR (SEQ ID NO:24) selected from:

a. Nucleotides 1 to 18 (ATGGGAGAGAAGGAGGGC);
b. Nucleotides 205 to 222 (CTGGTCCGTTACATTTGT);
c. Nucleotides 247 to 264 (GTGGCTCCCGGTGAGAGG);
d. Nucleotides 298 to 315 (GACTGGCTGTACACTTTC);
e. Nucleotides 321 to 338 (GAGGCCGGAGGTGGTGCA);
f. Nucleotides 351 to 368 (AGATCCCCCGAGACCTCA);

g. Nucleotides 379 to 396 (ATGAATGAGGCCAAGGTG);
h. Nucleotides 511 to 528 (AGTTGGAGTTCATTGGAT); and
i. Nucleotides 531 to 548 (GCGGCGGGAAAGTGGCTC).

[0100] In a further aspect, the invention provides a method of treating or inhibiting the progression of cancer in a mammal, comprising administering to a mammal a therapeutically effective amount of an antisense oligonucleotide, wherein the antisense oligonucleotide comprises a sequence specifically hybridizable to a portion of the coding region of human KSR (SEQ ID NO:24) selected from:

a. Nucleotides 1 to 18 (ATGGGAGAGAAGGAGGGC);
b. Nucleotides 205 to 222 (CTGGTCCGTTACATTTGT);
c. Nucleotides 247 to 264 (GTGGCTCCCGGTGAGAGG);
d. Nucleotides 298 to 315 (GACTGGCTGTACACTTTC);
e. Nucleotides 321 to 338 (GAGGCCGGAGGTGGTGCA);
f. Nucleotides 351 to 368 (AGATCCCCCGAGACCTCA);
g. Nucleotides 379 to 396 (ATGAATGAGGCCAAGGTG);
h. Nucleotides 511 to 528 (AGTTGGAGTTCATTGGAT); and
i. Nucleotides 531 to 548 (GCGGCGGGAAAGTGGCTC).

Cancers which are susceptible to the invention's method include cancer selected from the group of pancreatic cancer, lung cancer, skin cancer, urinary tract cancer, bladder cancer, liver cancer, thyroid cancer, colon cancer, intestinal cancer, leukemia, lymphoma, neuroblastoma, head and neck cancer, breast cancer, ovarian cancer, stomach cancer, esophageal cancer and prostate cancer.

[0101] The invention provides a method for sensitizing hyperproliferative, cancer or tumor cells in a mammal to ionizing radiation or other radiation or chemotherapy comprising administering to a mammal a therapeutically effective amount of one or more inhibitor of KSR expression and/or activity. In particular, the invention provides a method of conferring radiosensitivity to ionizing radiation in tumor cells in a mammal comprising administering to a mammal a therapeutically effective amount of a compound or agent which inhibits the expression of mammalian KSR protein.

[0102] In particular, such a method comprises administering to a mammal a therapeutically effective amount of an antisense oligonucleotide, wherein the antisense oligonucleotide is specifically hybridizable to a portion of the coding region of mRNA encoding human KSR (SEQ ID NO:24).

[0103] In a yet further aspect, the invention provides a method for modulating angiogenesis in a mammal by modulation of VEGF expression upon activation or inhibition of expression and/or activity of KSR. Inhibition, blockage or reduction of KSR decreases the amount or expression of VEGF, thereby having an anti-angiogenic effect. In a particular such method, VEGF expression of

2004297594 16 Sep 2009

cancer or tumor cells is blocked by inhibition of KSR, including by administration or expression of antisense oligonucleotide complementary to KSR, resulting in inhibition of angiogenesis in the cancer or tumor. In particular, the invention provides a method of inhibiting angiogenesis of a tumor in a mammal comprising administering to a mammal a therapeutically effective amount of a compound or agent which inhibits the expression of mammalian KSR protein.

[0104(a)] In particular, such a method comprises administering to a mammal a therapeutically effective amount of an antisense oligonucleotide, wherein the antisense oligonucleotide is specifically hybridizable to a portion of the coding region of mRNA encoding human KSR (SEQ ID NO:24).

[0104(b)] In a particular aspect, the oligonucleotides may be modified, either by manipulation of the chemical backbone of the nucleic acids or by covalent or non-covalent attachment of other moieties. In each or any case, such manipulation or attachment may serve to modify the stability, cellular, tissue or organ uptake, or otherwise enhance efficacy of the oligonucleotides covalently linked to other molecules, including but not limited to polypeptides, carbohydrates, lipid or lipid-like moieties, ligands, chemical agents or compounds, which may serve to enhance the uptake, stability or to target the oligonucleotides.

[0105] In further embodiments, the oligonucleotides are modified in their chemical backbone. In a particular embodiment, the oligonucleotides comprise at least one phosphorothioate linkage.

[0106] The oligonucleotides of the present invention may be combined with oligonucleotides directed to other targets, by mixture or by non-covalent or covalent attachment. For instance, the KSR antisense oligonucleotides may be combined with antisense directed to raf as described in U.S. Patent 6,391,636 (incorporated herein by reference) or to other oncogenic or proliferative proteins.

[0107] Recombinant DNA molecules comprising a nucleic acid sequence which encodes on transcription an antisense RNA complementary to mammalian KSR RNA or a portion thereof are provided by the invention. Further, the recombinant DNA molecules comprise a nucleic acid sequence wherein said nucleic acid sequence is operatively linked to a transcription control sequence. Cell lines transfected with these recombinant DNA molecules are also included in the invention.

[0108] In a further aspect, an expression vector is provided which is capable of expressing a nucleic acid which is substantially complementary to the coding sequence of KSR RNA, or a portion thereof, wherein said nucleic acid inhibits the expression of KSR. In a particular aspect, this includes an expression vector capable of expressing an oligonucleotide which is substantially complementary to the CA1 region of the coding sequence of KSR RNA, or a portion thereof, wherein said oligonucleotide inhibits the expression of KSR.

[0109] In an additional embodiment, the invention provides a composition comprising an expression vector and a pharmaceutically acceptable carrier or diluent, wherein said expression vector is capable of expressing nucleic acid which is substantially complementary to the coding sequence of KSR RNA, or a portion thereof, wherein said nucleic acid inhibits the expression of KSR.

[0110] The present invention naturally contemplates several means for preparation of the agents and antisense oligonucleotides, including as illustrated herein known recombinant techniques, and the invention is accordingly intended to cover such synthetic preparations within its scope. The knowledge of the cDNA and amino acid sequences of KSR as disclosed herein facilitates the preparation of the nucleic acids of the invention by such recombinant techniques, and accordingly, the invention extends to

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expression vectors prepared from the disclosed DNA sequences for expression in host systems by recombinant DNA techniques, and to the resulting transformed hosts.

[0111] Another feature of this invention is the expression of the nucleic acids disclosed herein. As is well known in the art, nucleic acid or DNA sequences may be expressed by operatively linking them to an expression control sequence in an appropriate expression vector and employing that expression vector to transform an appropriate unicellular host. Such operative linking of a nucleic acid sequence of this invention to an expression control sequence, of course, includes, if not already part of the DNA sequence, the provision of an initiation codon, ATG, in the correct reading frame upstream of the DNA sequence.

[0112] A wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, e.g., *E. coli* plasmids col El, pCR1, pBR322, pMB9 and their derivatives, plasmids such as RP4; phage DNAS, e.g., the numerous derivatives of phage λ, e.g., NM989, and other phage DNA, e.g., M13 and filamentous single stranded phage DNA; yeast plasmids such as the 2μ plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like. In addition, viral and retroviral vectors, including but not limited to adenovirus and adeno-associated virus, may be useful in such expression.

[0113] Any of a wide variety of expression control sequences -- sequences that control the expression of a DNA sequence operatively linked to it -- may be used in these vectors to express the DNA sequences of this invention. Such useful expression control sequences include, for example, the early or late promoters of SV40, CMV, vaccinia, polyoma or adenovirus, the *lac* system, the *trp* system, the *TAC* system, the *TRC* system, the *LTR* system, the major operator and promoter regions of phage λ, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase (e.g., Pho5), the promoters of the yeast - mating factors, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

[0114] A wide variety of unicellular host cells are also useful in expressing the DNA sequences of this invention. These hosts may include well known eukaryotic and prokaryotic hosts, such as strains of *E. coli*, *Pseudomonas*, *Bacillus*, *Streptomyces*, fungi such as yeasts, and animal cells, such as CHO, Rl.l, B-W and L-M cells, African Green Monkey kidney cells (e.g., COS 1, COS 7, BSC1, BSC40, and BMT10), insect cells (e.g., Sf9), and tumor cells, transformed cells, human cells and plant cells in tissue culture.

[0115] It will be understood that not all vectors, expression control sequences and hosts will function equally well to express the DNA sequences of this invention. Neither will all hosts function equally well with the same expression system. However, one skilled in the art will be able to select the proper vectors, expression control sequences, and hosts without undue experimentation to accomplish the desired expression without departing from the scope of this invention. For example, in selecting a vector, the host must be considered because the vector must function in it. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, will also be considered.

[0116] In selecting an expression control sequence, a variety of factors will normally be considered. These include, for example, the relative strength of the system, its controllability, and its compatibility with the particular DNA sequence or gene to be expressed, particularly as regards potential secondary structures. Suitable unicellular hosts will be selected by consideration of, e.g., their compatibility with the chosen vector, their secretion characteristics, their ability to fold proteins correctly, and their fermentation requirements, as well as the toxicity to the host of the product encoded by the DNA sequences to be expressed, and the ease of purification of the expression products. Considering these and other factors a person skilled in the art will be able to construct a variety of vector/expression control sequence/host combinations that will express the DNA sequences of this invention on fermentation or in large scale animal culture.

[0117] The present invention further includes transgenic animals and animal models wherein KSR is knocked out or otherwise nullified (as in the *ksr*$^{-/-}$ animals described herein) or wherein KSR is overexpressed as further described herein. Such animal models include mammals for instance mice, rats, pigs, rabbits, dogs, monkeys, etc. and any other recognized vertebrate or invertebrate system for study, including ducks, fish, drosphila, *C. elegans*, etc. In the case of nullified KSR, these animals are useful for the study of oncogenesis or the blockage of tumorigenesis in a KSR null background, including to identify other factors in tumorigenesis or metastasis. In the case of KSR overexpressors, wherein tumorigenesis, cell proliferation and metastasis is enhanced, these systems may be useful for the rapid study of tumor models and for evaluating potential anti-cancer compounds or agents, including those targeting KSR as well as other pathways.

[0118] As mentioned above, nucleic acids and oligonucleotides of the present invention can be prepared synthetically rather than cloned. In general, one will select preferred codons for the intended host if the sequence will be used for expression. The complete sequence is assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge, *Nature*, 292:756 (1981); Nambair et al., *Science*, 223:1299 (1984); Jay et al., *J. Biol. Chem.*, *259*:6311 (1984).

[0119] Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule. (See Weintraub, 1990; Marcus-Sekura, 1988.) In the cell, they hybridize to that mRNA, forming a double stranded molecule and interfering with the expression of mRNA into protein. Antisense methods have been used to inhibit the expression of many genes *in vitro* (Marcus-Sekura, 1988; Hambor et al., 1988).

[0120] The antisense oligonucleotides of the invention are selected as substantially complementary to a region of KSR mRNA. The oligonucleotides of the present invention may be complementary to regions including but not limited to: a) the 5'-cap site of an mRNA molecule (Ojala et al. (1997) Antisense Nucl. Drug Dev. 7:31-38); b) the transcription start site (Monia et al. (1992) J. Biol. Chem. 267:19954-19962); c) the translation initiation codon (Dean et al. (1994) Proc. Natl. Acad. Sci. U.S.A. 91:11762-11766); d) the translation stop codon (Wang et al. (1995) Proc. Natl. Acad. Sci. USA 92:3318-3322); e) mRNA splice sites (Agrawal et al. (1988) Proc. Natl. Acad. Sci. U.S.A. 86:7790-7794; Colige et al. (1993) Biochem. 32:7-11); f) the 5'-untranslated region of mRNA molecules (Duff et al. (1995) J. Biol. Chem. 270:7161-7166; Yamagami et al. (1996) Blood 87:2878-2884); g) the 3'-untranslated region of mRNA molecules (Bennett et al. (1994) J. Immunol. 152:3530-3540; Dean et al. (1994) J. Biol. Chem. 269:16146-16424); and h) the coding region (Laptev et al. (1994) Biochem. 33:11033-11039; Yamagami et al. (1996) Blood 87:2878-2884).

[0121] The skilled artisan can readily utilize any of several strategies to facilitate and simplify the selection process for nucleic acids and oligonucleotides effective in inhibition of KSR expression. Predictions of the binding energy or calculation of thermodynamic indices between an olionucleotide and a complementary sequence in an mRNA molecule may be utilized (Chiang et al. (1991) J. Biol. Chem. 266:18162-18171; Stull et al. (1992) Nucl. Acids Res. 20:3501-3508). Antisense oligonucleotides may be selected on the basis of secondary structure (Wickstrom et al (1991) in Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS, Wickstrom, ed., Wiley-Liss, Inc., New York, pp. 7-24; Lima et al. (1992) Biochem. 31:12055-12061). Schmidt and Thompson (U.S. Patent 6416,951) describe a method for identifying a functional antisense agent comprising hybridizing an RNA with an oligonucleotide and measuring in real time the kinetics of hybridization by hybridizing in the presence of an intercalation dye or incorporating a label and measuring the spectroscopic properties of the dye or the label's signal in the presence of unlabelled oligonucleotide. In addition, any of a variety of computer programs may be utilized which predict suitable antisense oligonucleotide sequences or antisense targets utilizing various criteria recognized by the skilled artisan, including for example the absence of self-complementarity, the absence hairpin loops, the absence of stable homodimer and duplex formation (stability being assessed by predicted energy in kcal/mol). Examples of such computer programs are readily available and known to the skilled artisan and include the OLIGO 4 or OLIGO 6 program (Molecular Biology Insights, Inc., Cascade, CO) and the Oligo Tech program (Oligo Therapeutics Inc., Wilsonville, OR).

[0122] In addition, antisense oligonucleotides suitable in the present invention may be identified by screening an oligonucleotide library, or a library of nucleic acid molecules, under hybridization conditions and selecting for those which hybridize to the target RNA or nucleic acid (see for example U.S. Patent 6,500,615). Mishra and Toulme have also developed a selection procedure based on selective amplification of oligonucleotides that bind target (Mishra et al (1994) Life Sciences 317:977-982). Oligonucleotides may also be selected by their ability to mediate cleavage of target RNA by RNAse H, by selection and characterization of the cleavage fragments (Ho et al (1996) Nucl Acids Res 24:1901-1907; Ho et al (1998) Nature Biotechnology 16:59-630). Generation and targeting of oligonucleotides to GGGA motifs of RNA molecules has also been described (U.S. Patent 6,277,981).

[0123] Inhibition of *ksr* gene expression can be measured in ways which are routine in the art, for example by Northern blot assay of mRNA expression or Western blot assay of protein expression as well known to the skilled artisan. Effects on cell proliferation or tumor cell growth can also be measured, *in vitro* or *in vivo*, in cell, tumor or animal model systems, by methods well known to the skilled artisan, including as taught in the examples of the instant application. Similarly, inhibition of KSR activity, particularly phosphorylation or kinase activity may be measured.

[0124] "Substantially complementary" is used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between the DNA or RNA target and the oligonucleotide or nucleic acid. It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target interferes with the normal function of the target molecule to cause a loss of utility or expression, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under physiological conditions in the case of *in vivo* assays or therapeutic treatment or, in the case of *in vitro* assays, under conditions in which the assays are conducted.

[0125] In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages. Oligonucleotide includes oligomers comprising non-naturally occurring monomers, or portions thereof, which function similarly and such modified or substituted oligonucleotides may be preferred over native forms because of, for example, enhanced cellular uptake and increased stability against nucleases. The oligonucleotides of the present invention may contain two or more chemically distinct regions, each made up of at least one nucleotide, for instance, at least one region of modified nucleotides that confers one or more beneficial properties (for example, increased nuclease resistance, increased uptake into cells, increased binding affinity for the RNA target) and a region that is a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids (for example, RNase H - a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex).

[0126] In a preferred embodiment, the region of the oligonucleotide which is modified to increase KSR mRNA binding affinity comprises at least one nucleotide modified at the 2' position of the sugar, most preferably a 2'-O-alkyl, 2'-O-alkyl-O-alkyl or 2'-fluoro-modified nucleotide. Such modifications are routinely incorporated into oligonucleotides and these oligonucleotides have been shown to have a higher Tm (i.e., higher target binding affinity) than 2'-deoxyoligonucleotides against a given target. In another preferred embodiment, the oligonucleotide is modified to enhance nuclease resistance. Cells contain a variety of exo- and endo-nucleases which can degrade nucleic acids. A number of nucleotide and nucleoside modifications have been shown to confer relatively greater resistance to nuclease digestion. Oligonucleotides which contain at least one phosphorothioate modification are presently more preferred (Geary, R.S. et al (1997) Anticancer Drug Des 12:383-93; Henry, S. P. et al (1997) Anticancer Drug Des 12:395-408; Banerjee, D. (2001) Curr Opin Investig Drugs 2:574-80). In some cases, oligonucleotide modifications which enhance target binding affinity are also, independently, able to enhance nuclease resistance.

[0127] Specific examples of some preferred oligonucleotides envisioned for this invention include those containing modified backbones, for example, phosphorothioates, phosphotriesters, methyl phosphonates, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. Most preferred are oligonucleotides with phosphorothioate backbones and those with heteroatom backbones. The amide backbones disclosed by De Mesmaeker et al. (1995) Acc. Chem. Res. 28:366-374) are also preferred. Also preferred are oligonucleotides having morpholino backbone structures (Summerton and Weller, U.S. Pat. No. 5,034,506). In other preferred embodiments, such as the peptide nucleic acid (PNA) backbone, the phosphodiester backbone of the oligonucleotide is replaced with a polyamide backbone, the nucleobases being bound directly or indirectly to the aza nitrogen atoms of the polyamide backbone (Nielsen et al., Science, 1991, 254, 1497). Oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH, SH, SCH_3, F, OCN, heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted silyl; an RNA cleaving group; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide and the 5' position of 5' terminal nucleotide.

[0128] Oligonucleotides may also include, additionally or alternatively base modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include adenine (A), guanine (G), thymine (T), cytosine (C) and uracil (U). Modified nucleobases include nucleobases found only infrequently or transiently in natural nucleic acids, e.g., hypoxanthine, 6-methyladenine, 5-me pyrimidines, particularly 5-methylcytosine (5-me-C) (Sanghvi, Y. S., in Crooke, S. T. and Lebleu, B., eds., Antisense Research and Applications, CRC Press, Boca Raton, 1993, pp. 276-278), 5-hydroxymethylcytosine (HMC), glycosyl HMC and gentobiosyl HMC, as well as synthetic nucleobases, including but not limited to, 2-aminoadenine, 2-thiouracil, 2-thiothymine, 5-bromouracil, 5-hydroxymethyluracil, 8-azaguanine, 7-deazaguanine (Kornberg, A., DNA Replication, W.H. Freeman & Co., San Francisco, 1980, pp75-77; Gebeyehu, G., et al., 1987, Nucl. Acids Res. 15:4513). A "universal" base known in the art, e.g., inosine, may be included.

[0129] Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety, a cholesteryl moiety (Letsinger et al. (1989) Proc. Natl. Acad. Sci. USA 86: 6553), cholic acid (Manoharan et al. (1994) Bioorg. Med. Chem. Let. 4:1053), a thioether, for example, hexyl-S-tritylthiol (Manoharan et al. (1992) Ann. N.Y. Acad. Sci. 660: 306; Manoharan et al. (1993) Bioorg. Med. Chem. Let. 3: 2765), a thiocholesterol (Oberhauser et al. (1992) Nucl. Acids Res.20:533), an aliphatic chain, for example, dodecandiol or undecyl residues (Saison-Behmoaras et al. (1991) EMBO J. 10:111; Kabanov et al. (1990) FEBS Lett. 259:327; Svinarchuk et al. (1993) Biochimie 75:49), a phospholipid, a polyamine or a polyethylene glycol chain (Manoharan et al. (1995) Nucleosides & Nucleotides 14:969). Oligonucleotides comprising lipophilic moieties, and methods for preparing such oligonucleotides are known in the art, for example, U.S. Pat. Nos. 5,138,045, 5,218,105 and 5,459,255.

[0130] Farrel and Kloster (U.S. Patent 6,310,047) describe the enhancement of delivery and of *in vivo* nuclease resistance of antisense oligonucleotides using high affinity DNA binding polynuclear platinum compounds.

[0131] It is not necessary for all positions in a given oligonucleotide to be uniformly modified, and more than one of the aforementioned modifications may be incorporated in a single oligonucleotide or even at a single nucleoside within an oligonucleotide.

[0132] The oligonucleotides in accordance with this invention preferably are from about 8 to about 50 nucleotides in length. Particularly preferred oligonucleotides are from 10 to 30 nucleotides in length, particularly preferred are from 15 to 25 nucleotides. In the context of this invention it is understood that this encompasses non-naturally occurring oligomers as hereinbefore described, having 8 to 50 monomers.

[0133] The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis may also be employed; the actual synthesis of the oligonucleotides is well within the talents of the skilled artisan. It is also well known to use similar techniques to prepare other oligonucleotides such as the phosphorothioates and alkylated derivatives.

[0134] The therapeutic possibilities that are raised by the methods and compositions, particularly oligonucleotides, of the present invention derive from the demonstration in the Examples herein that inactivation of KSR, including by genetic knockout, by inhibition of its expression utilizing antisense oligonucleotides, and by expression of reverse complement RNA or antisense DNA constructs, results in specific blockage of Ras-mediated tumorigenesis and cellular hyperproliferation, including via *gf*-Ras, and treatment or inhibition of progression of cancer. The present invention contemplates pharmaceutical intervention in the cascade of reactions in which overexpressed or amplified, hyperactivated or oncogenic Ras, including *gf*-Ras, is implicated, to regress, block, treat or inhibit the progression of Ras-mediated tumors, oncogenesis, metastasis and angiogenesis. Thus, in instances where it is desired to reduce or inhibit Ras, including *gf*-Ras, the nucleic acids and oligonucleotides of the present invention could be introduced to block or inhibit the Ras pathway.

[0135] The invention further includes a method of treating or preventing a hyperproliferative condition associated with the expression of *gf*-Ras or heightened expression or hyperactivation of Ras or the Ras pathway in a mammal comprising administering to said mammal a therapeutically effective amount of a compound or agent which inhibits the expression or activity of mammalian KSR protein. In one aspect of this method, said compound or agent is an antisense oligonucleotide which specifically hybridizes to a portion of the mRNA encoding KSR. A method of treating or preventing a hyperproliferative condition associated with the expression of gf-Ras or heightened expression or hyperactivation of Ras in a mammal is provided, comprising expressing in said mammal or administering to said mammal a therapeutically effective amount of a nucleic acid which is complementary to a portion of the mRNA encoding KSR.

[0136] The invention further provides a method for sensitizing hyperproliferative, cancer or tumor cells in a mammal to ionizing radiation or other radiation or chemotherapy comprising administering to a mammal a therapeutically effective amount of one or more inhibitor of KSR expression and/or activity. The invention provides a method for sensitizing hyperproliferative, cancer or tumor cells in a mammal to ionizing radiation or other radiation or chemotherapy comprising administering to a mammal a therapeutically effective amount of one or more oligonucleotide of the present invention.

[0137] The present invention further provides a method for modulating angiogenesis, particularly by modulation of VEGF expression upon activation or inhibition of expression and/or activity of KSR. VEGF expression is modulated by specific inhibition or activation of KSR expression and/or activity. Activation or enhanced expression of KSR increases the amount or expression of VEGF in or by cells. Thus, activation or enhanced expression of KSR in a cell or tissue provides a method of stimulating angiogenesis. Inhibition, blockage or reduction of KSR decreases the amount or expression of VEGF, thereby having an anti-angiogenic effect. In one such aspect, VEGF expression of cancer or tumor cells is blocked by inhibition of KSR, including by administration or expression of antisense oligonucleotide complementary to KSR, resulting in inhibition of angiogenesis in the cancer or tumor. Inhibition of KSR, including by administration or expression of antisense oligonucleotide complementary to KSR, provides a method of inhibiting angiogenesis in a tumor, tissue or cells expressing KSR, particularly a tissue or tumor expressing *gf* Ras or wherein the Ras pathway is hyperactivated or Ras is overexpressed or amplified.

[0138] In a further aspect, a method of treating or inhibiting the progression of cancer in a mammal is included, comprising administering to a mammal a therapeutically effective amount of a compound or agent which inhibits the expression or activity of mammalian KSR protein. Cancers which are susceptible to the invention's method include cancer selected from the group of pancreatic cancer, lung cancer, skin cancer, urinary tract cancer, bladder cancer, liver cancer, thyroid cancer, colon cancer, intestinal cancer, leukemia, lymphoma, neuroblastoma, head and neck cancer, breast cancer, ovarian cancer, stomach cancer, esophageal cancer and prostate cancer. In one aspect, a method of treating or inhibiting the progression of pancreatic cancer in a mammal is provided, comprising administering to a mammal a therapeutically effective amount of a compound or agent which inhibits the expression or activity of mammalian KSR protein.

[0139] Thus, a method is provided for treating or inhibiting the progression of cancer in a mammal comprising administering to a mammal a therapeutically effective amount of one or more oligonucleotide of the present invention. A method is provided for treating or inhibiting the progression of pancreatic cancer in a mammal comprising administering to a mammal a therapeutically effective amount of one or more oligonucleotide of the present invention.

[0140] The present invention further contemplates therapeutic compositions useful in practicing the therapeutic methods of this invention. A subject therapeutic composition includes, in admixture, a pharmaceutically acceptable carrier (excipient) or diluent and one or more nucleic acid or oligonucleotide of the invention as described herein as an active ingredient. In a preferred embodiment, the composition comprises an oligonucleotide capable of inhibiting the expression of KSR.

[0141] Compositions of the nucleic acids and oligonucleotides are an additional aspect of the invention. The invention includes a composition comprising an oligonucleotide which is substantially complementary to a region of KSR RNA and a pharmaceutically acceptable carrier or diluent. The invention thus provides a pharmaceutical composition comprising a therapeutically effective amount of an oligonucleotide which is substantially complementary to a region of KSR RNA and a pharmaceutically acceptable carrier or diluent.

[0142] In a further aspect, compositions are provided comprising one or more chemotherapeutic or radiotherapeutic agent and an oligonucleotide which is targeted to a mRNA encoding mammalian KSR and which inhibits KSR expression.

[0143] The preparation of therapeutic compositions which contain nucleic acids, oligonucleotides, or analogs as active ingredients is well understood in the art. Such compositions can be prepared as injectables, either as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The compositions may be prepared in solid pill form, including slow release formulations. The composition may be in a patch form for transdermal application, particularly in slow release format. The preparation can also be emulsified. The active therapeutic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents which enhance the effectiveness of the active ingredient.

[0144] A nucleic acid or oligonucleotide can be formulated into the therapeutic composition as neutralized pharmaceutically acceptable salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide or antibody molecule) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

[0145] The therapeutic nucleic acid-, oligonicleotide-, analog- or active fragment-containing compositions may be administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for humans, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

[0146] The therapeutic compositions may further include an effective amount of the nucleic acid or oligonucleotide, and one or more of the following active ingredients or agents: a chemotherapeutic agent, a radiotherapeutic agent, an immunomodulatory agent, an anti-mitotic agent.

[0147] The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, transdermal), oral or parenteral. Parenteral administration includes intravenous drip or infusion, subcutaneous, intraperitoneal or intramuscular injection, pulmonary administration, e.g., by inhalation or insufflation, or intrathecal or intraventricular administration. For oral administration, it has been found that oligonucleotides with at least one 2'-substituted ribonucleotide are particularly useful because of their absorption and distribution characteristics. U.S. Pat. No. 5,591,721 (Agrawal et al.) and may be suitable for oral administration. Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful. Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. Compositions for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives. In addition to such pharmaceutical carriers, cationic lipids may be included in the formulation to facilitate oligonucleotide uptake. One such composition shown to facilitate uptake is Lipofectin (BRL Bethesda Md.).

[0148] Dosing is dependent on severity and responsiveness of the condition to be treated, with course of treatment lasting from several days to several months or until a cure is effected or a diminution of disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be calculated based on IC50s or EC50s in *in vitro* and *in vivo* animal studies. For example, given the molecular weight of compound (derived from oligonucleotide sequence and chemical structure) and an effective dose such as an IC50, for example (derived experimentally), a dose in mg/kg is routinely calculated.

[0149] The oligonucleotides of the invention are also useful for detection and diagnosis of KSR expression. For example, radiolabeled oligonucleotides can be prepared by radioactive (e.g. ^{32}P) labeling at the 5' end or 3' end (including with polynucleotide kinase), contacted with tissue or cell samples suspected of KSR expression or of gf-Ras and unbound oligonucleotide removed. Radioactivity remaining in the sample indicates bound oligonucleotide (which in turn indicates the presence of KSR or of gf-Ras) and can be quantitated using a scintillation counter or other routine means. Radiolabeled oligonucleotide can also be used to perform autoradiography of tissues to determine the localization, distribution and quantitation of KSR or gf-Ras expression for research, diagnostic or therapeutic purposes. In addition, the radiolabel may have a therapeutic effect in promoting cell death or blocking cellular proliferation. Analogous assays for fluorescent detection of raf expression can be developed using oligonucleotides of the invention which are conjugated with fluorescein or other fluorescent tag instead of radiolabeling.

[0150] The oligonucleotides of the present invention may be labeled with a detectable label. In particular aspects, the label may be selected from enzymes, chemicals which fluoresce and radioactive elements. In the instance where a radioactive label, such as the isotopes ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{36}Cl, ^{51}Cr, ^{57}Co, ^{58}Co, ^{59}Fe, ^{90}Y, ^{125}I, ^{131}I, and ^{186}Re are used, known currently available counting procedures may be utilized. In the instance where the label is an enzyme, detection may be accomplished by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques known in the art. A number of fluorescent materials are known and can be utilized as labels. These include, for example, fluorescein, rhodamine, auramine, Texas Red, AMCA blue and Lucifer Yellow. A particular detecting material is anti-rabbit antibody prepared in goats and conjugated with fluorescein through an isothiocyanate. Enzyme labels are also useful, and can be detected by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques. The enzyme is conjugated to the selected particle by reaction with bridging molecules such as carbodiimides, diisocyanates, glutaraldehyde and the like. Many enzymes which can be used in these procedures are known , including but not limited to peroxidase, ß-glucuronidase, ß-D-glucosidase, ß-D-galactosidase, urease, glucose oxidase plus peroxidase and alkaline phosphatase. U.S. Patent Nos. 3,654,090; 3,850,752; and 4,016,043 are referred to by way of example for their disclosure of alternate labeling material and methods.

[0151] The invention includes additional compositions which can inhibit the expression of a protein, in particular KSR, at the transcriptional level by blocking translation of KSR mRNA or by facilitating destruction or destabilization of the RNA such that translation cannot efficiently take place. In this aspect, the invention provides a ribozyme that cleaves KSR mRNA.

[0152] Ribozymes are RNA molecules possessing the ability to specifically cleave other single stranded RNA molecules in a manner somewhat analogous to DNA restriction endonucleases. Ribozymes were discovered from the observation that certain mRNAs have the ability to excise their own introns. By modifying the nucleotide sequence of these RNAs, researchers have been able to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech, 1988.). Because they are sequence-specific, only mRNAs with particular sequences are inactivated.

[0153] Investigators have identified two types of ribozymes, *Tetrahymena*-type and "hammerhead"-type. (Hasselhoff and Gerlach, 1988) *Tetrahymena*-type ribozymes recognize four-base sequences, while "hammerhead"-type recognize eleven- to eighteen-base sequences. The longer the recognition sequence, the more likely it is to occur exclusively in the target mRNA species. Therefore, hammerhead-type ribozymes are preferable to *Tetrahymena*-type ribozymes for inactivating a specific mRNA species, and eighteen base recognition sequences are preferable to shorter recognition sequences.

[0154] The use of RNA interference strategies to inhibit the expression of KSR is further embodied in the invention. Thus, methods of RNA interference and small interfering RNA compositions are included in the methods and compositions of the present invention. RNA interference refers to the silencing of genes specifically by double stranded RNA (dsRNA) (Fine, A. et al (1998) Nature 391;806-811). In one embodiment, short or small interfering RNA (siRNA) is utilized (Elbashir, S.M. et al (2001) Nature 411:494-498). In addition, long double stranded RNA hairpins may be employed (Tavernarakis, N. et al (2000) Nature Genet 24:180-183; Chuang, C.F. and Meyerowitz, E.M. (2000) PNAS USA 97:4985-90; Smith, NA et al (2000) Nature 407:319-20). Virus-mediated RNA interference against K-Ras has been described (B rummelkamp, T.R. et al (2002) Cancer Cell 2:243-247).

[0155] The invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention. The following examples are presented in order to more fully illustrate the preferred embodiments of the invention and should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLES

[0156] These studies demonstrate that mammalian KSR integrates signaling through the EGFR/Ras/MAPK signaling module. That EGFR, Ras and KSR are on the same signaling pathway in mammalian cells is supported by the unusual hair follicle phenotype manifested in EGFR knockout mice and recapitulated in the KSR knockout, by the attenuation of EGF-induced MAPK signaling in MEFs, and by the abrogation of EGFR-/Ras-mediated tumorigenesis in multiple experimental models. Further, genetic and pharmacologic approaches identified KSR as required for various aspects of tumorigenesis *in vitro* and *in vivo*. *In vitro*, loss of KSR function reduced proliferation of MEFs, A431 and MCF-7 cells, abrogated Ras-mediated MEF transformation, and attenuated A431 and MCF-7 cell invasion. *In vivo*, inactivation of KSR antagonized *v-*Ha-*Ras*-mediated tumor formation and growth of an established EGFR-driven tumor that requires wild type Ras for neoplastic progression. As in *C. elegans*[2,3], KSR appears dispensable, for the most part, for normal development, but required when increased signaling through the EGFR/Ras pathway is necessary, as occurs acutely in response to EGF stimulation or chronically in Ras-mediated tumors. This suggests that pharmacologic inactivation might yield a therapeutic gain. Indeed, the results presented herein, including *in vivo* models of Ras-mediated tumorigenesis, show significant inhibition of cell proliferation and cell invasion on inactivation of *ksr*. These studies demonstrate the use of *ksr* antisense oligonucleotides (KSR-AS ODNs) as a therapeutic approach in cancer and tumorigenesis, particularly K-Ras mediated tumorigenesis.

EXAMPLE 1

ABSTRACT

[0157] In *Drosophila melanogaster* and *Caenorhabditis elegans,* Kinase Suppressor of Ras (KSR) positively modulates Ras/mitogen-activated protein kinase (MAPK) signaling either upstream of or parallel to Raf[1-3]. The precise signaling mechanism of mammalian KSR, and its role in Ras-mediated transformation, however, remains uncertain. Utilizing cells markedly overexpressing recombinant KSR, some groups reported KSR inhibits MAPK activation and Ras-induced transformation[4-6] while others observed enhancing effects[7-10]. Evidence suggests these discrepancies reflect gene dosage effects[11]. To gain insight into KSR function *in vivo*, we generated mice homozygous null for KSR. $ksr^{-/-}$ mice are viable and without major developmental defects. Newborn mice, however, display a unique hair follicle phenotype previously observed in EGFR-deficient mice, providing genetic support for the notion that EGFR, Ras and KSR are on the same signaling pathway in mammals. Embryonic fibroblasts from $ksr^{-/-}$ animals were defective in EGF activation of the MAPK pathway, and displayed diminished proliferative potential and impaired Ras-dependent transformability. Tumor formation in Tg.AC mice, resulting from skin-specific *v*-Ha-*ras* expression, was abrogated in the $ksr^{-/-}$ background. Thus, evidence presented herein suggests KSR transduces EGFR-/Ras-mediated neoplasia, which may be potentially targeted by anti-KSR therapeutic strategies.

INTRODUCTION

[0158] Kinase suppressor of Ras (KSR) was identified in *Drosophiula melanogaster* and *Caenorhabditis elegans* as a positive modulator of Ras/mitogen-activated protein kinase (MAPK) signaling either upstream of or parallel to Raf (1-3). Although an intensive effort has been directed at elucidating the biochemical properties of mammalian KSR, its precise signaling mechanism remains uncertain. In particular, its role in Ras-mediated transformation has not been addressed convincingly. Some groups reported KSR inhibits MAPK activation and Ras-induced transformation (4-6) while others observed enhancing effects (8-10). These experiments utilized cell systems overexpressing recombinant KSR to levels far beyond endogenous KSR, and evidence suggests these discrepancies might reflect gene dosage (11). While we and others argue the necessity of both the kinase and scaffolding functions of KSR for its optimal activation of the Raf-MAPK cascade (26-30), others believe that KSR signals solely via its scaffolding function (9, 12, 31).

[0159] To gain insight into the *in vivo* function of KSR, we generated a mouse homozygous null for KSR. *ksr*$^{-/-}$ mice are viable and without major developmental defects. Newborn mice, however, display a unique hair follicle phenotype previously observed in EGFR-deficient mice. Mouse embryonic fibroblasts (MEFs) from *ksr*$^{-/-}$ animals displayed diminished proliferative potential and impaired oncogenic *v*-Ha-Ras-dependent transformation. Moreover, EGF and TPA activated the MAPK cascade to a similar extent in MEFs, yet only c-Raf-1 activation by mitogenic doses of EGF depended on *ksr*. The KSR knockout mouse thus allows the delineation of KSR-dependent and independent mechanisms of c-Raf-1 activation. Further, tumor formation in Tg.AC mice resulting from skin-specific *v*-Ha-*ras* expression, which utilizes MAPK signaling for transformation (32), was abrogated in the *ksr*$^{-/-}$ background. These defects in proliferation, transformation and tumor formation suggest KSR transduces some forms of Ras-mediated neoplasia.

RESULTS AND DISCUSSION

[0160] To investigate the *in vivo* function of KSR in mammals, we targeted the mouse *ksr* locus to obtain mice deficient in KSR expression. *ksr*$^{-/-}$ mice were generated by homologous recombination in embryonic stem (ES) cells using the pF9 targeting vector shown in FIGURE 1*a*. The targeted region included the starting methionine (ATG codon at nt 83 in *ksr* cDNA) and the following 74 amino acids encompassing 85% of the KSR unique CA1 domain. Two targeted ES clones (FIGURE 1*b*) were microinjected into

C57BL/6 blastocysts and both resulted in chimeric mice that transmitted the mutated *ksr* allele through to the germline. Crosses of the *ksr*$^{+/-}$ mice generated progeny with genotypes of the expected Mendelian frequencies. A PCR-based screening strategy was developed to detect both the wild-type (wt) and mutated alleles from mouse genomic DNA (FIGURE 1*c*).

[0161] As previously reported (12), Northern blot analysis revealed wt KSR transcripts of 6.4 and 7.4 kb. The smaller transcript was detected by embryonic day 7, while the larger transcript was observed from day 11 on (FIGURE 1*d*). In the adult, numerous tissues expressed *ksr* transcripts including heart, spleen, lung, thymus, and brain (FIGURE 1*e*). Kidney displayed little if any *ksr* mRNA, while the larger transcript was restricted to brain. The existence of this larger mRNA was recently reported by Morrison and co-workers to represent a splice variant of murine KSR1, named B-KSR1 (12). Importantly, *ksr*$^{-/-}$ mice did not express detectable levels of either *ksr* mRNA in any tissue tested (FIGURE 1*e*). KSR1 and B-KSR1 proteins were also not detected by Western blot analysis in tissues or in mouse embryo fibroblasts (MEFs) from *ksr*$^{-/-}$ mice (FIGURE 1*f*). The lack of KSR was also confirmed by RT-PCR analysis with primers specific for the 3'-UTR of *ksr* cDNA (not shown). Our data thus suggest that replacement of the 5' region of *ksr* including the start coding site and most of the CA1 domain successfully abolished expression of both murine KSR forms.

[0162] KSR knockout mice were viable and fertile, with no major developmental defects. No gross histologic abnormalities of the major organs were apparent in young mice or in adults up to one year of age. Animal weight, behavior and brood size were also unaffected in the KSR knockout. However, histologic examination of the skin of 10-day-old *ksr*$^{-/-}$ mice revealed noticeably fewer hair follicles, which were disorganized in dermal location (depth) and orientation (direction), and manifested asynchronous growth (FIGURE 2*a vs.* 2*b,c*). Further, a significant proportion displayed a serpentine morphology (FIGURE 2*b*). In other follicles, the inner root sheath separated from the hair shaft, resulting in formation of blisters or cysts (FIGURE 2*c*). Strikingly, this phenotype closely resembles that found in the skin of EGFR-deficient mice (13) (FIGURE 2*d*). Grossly, $egfr^{-/-}$ mice display short, wavy pelage hair and curly whiskers during the first weeks of age, with pelage and vibrissa hairs becoming progressively sparser and atrophic over time, eventually leading to alopecia (13). Although these gross phenotypes were not seen in $ksr^{-/-}$ mice, increased alopecia and sparse hair growth were observed following treatment with the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) compared to similarly treated $ksr^{+/+}$ controls (not shown). The manifestation of this unique hair follicle phenotype by both knockouts supports the contention that EGFR and KSR might be on the same pathway in mice.

[0163] To further elucidate the effect of KSR disruption on activation of the EGFR/MAPK pathway, we generated MEFs from $ksr^{+/+}$ and $ksr^{-/-}$ littermates and evaluated their response to low, mitogenic doses of EGF and TPA, two growth stimuli known to activate the MAPK cascade. After 48 hr of serum starvation, MAPK activation in response to various doses of EGF (0.01-100ng/ml) or TPA (10nM-1uM) was determined by Western blot analysis using the monoclonal and anti-phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) antibody. $ksr^{-/-}$ MEFs displayed a significant reduction in EGF- and TPA-induced MAPK (ERK1/2) activation at all doses examined, while total MAPK content remained largely unchanged (FIGURE 3A). For EGF stimulation, inhibition of MAPK activation was manifest at doses as low as 0.01 ng/ml (not shown), whereas at 100 ng/ml EGF, MAPK activation was partially restored (FIGURE 3A, upper panel, Lane 8).

[0164] To examine Raf-1 activation under conditions of MAPK inhibition, endogenous Raf-1 was evenly immunoprecipitated from all MEF lysates (not shown) and activity assayed using kinase-inactive MEK (K97M) as a substrate. While Raf-1 activity was greatly inhibited (>90%) in *ksr*$^{-/-}$ MEFs in response to mitogenic doses of EGF (FIGURE 3B, upper panel, lanes 4 and 6), no inhibition was observed when stimulated with 100 ng/ml EGF (FIGURE 3B, upper panel, lane 8). Thus, partial inhibition of MAPK activation in response to 100 ng/ml EGF in *ksr*$^{-/-}$ MEFs is independent of Raf-1 activation, likely resulting from the known MAK scaffolding function of KSR. These results indicate that EGF-stimulated Raf-1 activation in MEFs is dose-dependent and may occur via KSR-dependent and independent mechanisms, consistent with our previous findings (28).

[0165] The requirement for KSR for TPA-induced c-Raf-1 activation differed from that of mitogenic doses of EGF. In contrast to complete inhibition of c-Raf-1 activation after stimulation with mitogenic doses of EGF upon deletion of *ksr* , TPA-induced Raf-1 activation was not altered in *ksr*$^{-/-}$ MEFs (FIGURE 3B, lower panel). Thus, the use of the KSR knockout MEFs allows for the definition of two mechanisms of c-Raf-1 activation, a KSR-dependent mechanism necessary for mitogenic EGF stimulation, and a KSR-independent mechanism used by TPA, and perhaps pharmacologic doses of EGF. Loss of KSR thuis can impact M APK activation by two mechanisms, via loss of c-Raf-1 activation as well as the MEK scaffolding function of KSR.

[0166] To examine the biologic consequence of MAPK inhibition on cell proliferation *in vivo*, a proliferation assay was performed using MEFs in the exponential phase of cell growth. Consistent with reduction in signaling through the MAPK mitogenic pathway, which provides proliferative signals, a 50% reduction in growth rate in *ksr*$^{-/-}$ MEFS was observed (FIGURE 3C).

[0167] To determine the potential impact of KSR inactivation in Ras-mediated transformation, c-Myc and Ha-*ras*V12 constructs were transduced into *ksr*$^{+/+}$ and *ksr*$^{-/-}$ early-passage MEFs using high-titer retroviruses, and the ability to grow as colonies in soft agar was assessed as described (15). While $ksr^{+/+}$ fibroblasts did not form colonies in soft-agar, they did so in the presence of Myc and Ras oncogenes (not shown). In contrast, $ksr^{-/-}$ MEFs could not be transformed by Ha-*ras*V12, even though they were immortalized by c-Myc. Taken together, all these results show that inactivation of KSR by genetic deletion attenuates signaling through the EGFR/Ras/MAPK pathway.

[0168] The participation of oncogenic *ras* in human cancers is estimated to be 30% (33) and approximately 25% of skin lesions in humans involve mutations of the Ha-Ras (25% for squamous cell carcinoma and 28% for melanomas) (34,35). Since $ksr^{-/-}$ mice showed a defect in normal development of the hair follicle, presumably via impairment of EGFR signaling, we examined the role of KSR in gain-of-function Ras in the skin. For these studies, we employed Tg.AC mice, which harbor oncogenic *v*-Ha-*ras* fused to the ζ-globin promoter (16-18), a standardized model for the study of two-stage skin carcinogenesis. The *v*-Ha-*ras* transgene of Tg.AC mice is transcriptionally silent until induced in latent neoplastic cells (putative stem cells) closely associated with the outer root sheath cells of the hair follicle (19), a site consistent with our localization of KSR in mouse skin (not shown). Tg.AC mice (in FVB/N strain background) were crossed with $ksr^{-/-}$ mice (in a mixed C57BL/6:129sv background). F1 offspring heterozygous for the *ksr* gene were then interbred to obtain F2 offspring carrying the *v*-Ha-*ras* transgene in the $ksr^{+/+}$ and $ksr^{-/-}$ background. To determine if disruption of *ksr* might influence tumorigenesis in this model, we topically treated the dorsum of F2 mice twice weekly for 15 weeks with vehicle (acetone), or with 5 μg of TPA. Animals were monitored for development of skin malignancies for 20 weeks.

[0169] Initial control studies using RT-PCR to detect the *v*-Ha-*ras* transgene mRNA showed that loss of KSR function in $ksr^{-/-}$ mice had no impact on TPA-induced expression of the oncogenic *v*-Ha-*ras* transgene in the skin (FIGURE 4A). However, 70% of Tg.AC transgenic mice in a $ksr^{+/+}$ background developed papillomas, while only 10%

in a *ksr*$^{-/-}$ background displayed papillomas (FIGURE 4B). The average number of papillomas in our study was 2-4 per mouse in each group. These studies with Tg.AC mice demonstrate that genetic inactivation of KSR prevents EGFR-/Ras-mediated skin tumorigenesis.

[0170] In summary, these studies demonstrate that mammalian KSR integrates signaling through the EGFR/Ras/MAPK signaling module. That EGFR, Ras and KSR are on the same signaling pathway in mammalian cells is supported by the unusual hair follicle phenotype manifested in EGFR knockout mice and recapitulated in the KSR knockout, by the attenuation of EGF-induced MAPK signaling in MEFs, and by the abrogation of EGFR-/Ras-mediated tumorigenesis in multiple experimental models (see also Example 2). These studies further demonstrate that Raf-1 activation may occur by KSR-dependent and independent mechanisms. We believe this observation may help to resolve some of the questions regarding upstream elements of the Ras/Raf-1-MAPK module and provides new targets and reagents for additional investigation. In *C. elegans* (2,3), KSR appears dispensable, for the most part, for normal development, but required when increased signaling through the EGFR/Ras pathway is necessary and for some forms of oncogenic Ras-transduced MAPK-mediated tumorigenesis, as occurs acutely in response to EGF stimulation or chronically in Ras-mediated tumors, indicating that KSR inactivation could yield a therapeutic gain, particularly for selective abrogation of the Ras/MAPK signaling of human tumorigenesis.

METHODS

[0171] <u>Gene targeting.</u> Mouse *ksr* genomic DNA clones were isolated by screening a λFixII phage library prepared from mouse strain 129/sv (Stratagene, La Jolla, CA) using the 5' coding region (nt 1-786) of mouse *ksr* cDNA (Genbank accession # U43585). The mouse *ksr* cDNA sequence is provided in FIGURE 12A. The targeting vector pF9 was constructed by inserting a 2.5-kb *Spe*I-*Sma*I fill-in fragment from the 5' end of the mouse *ksr* genomic clone into the *Not*I fill-in site of pPGK-NTK vector (a gift from Dr. Frank Sirotnak). A 6.3-kb *Spe*I-*Hind*III fill-in fragment from the 3' downstream region of the mouse *ksr* genomic clone was inserted into the vector at the *Cla*I fill-in site. The resulting plasmid was linearized with *Kpn*I and electroporated into 129/Sv-derived W9.5 ES cells (Chrysalis DNX Transgenic Sciences, Princeton, New Jersey). Two hundred G418/Gancyclovir-resistant ES cell clones were analyzed by Southern blot using a 0.6 kb *Bgl*II-*Spe*I probe derived from genomic sequences located immediately outside (5') those present in pF9. This probe hybridizes to a 5.7-kb DNA fragment for the wt *ksr* allele and a 3.1-kb fragment from the disrupted allele. Heterozygous ES cells were microinjected into blastocyst-stage C57BL/6 mouse embryos at the Sloan-Kettering Institute's Transgenic Core Facility. Injected blastocysts were then transplanted into the uterus of pseudopregnant C57BL/6 mice. Chimeric males were crossed to C57BL/6 females. Germline transmission was monitored by Southern blot in agouti F1 offspring. For mouse genotyping, genomic DNA was isolated from mouse tails with the DNeasy kit (Qiagen Inc., Valencia, CA) and was either digested with *Bgl*II and *Xho*I and examined by Southern blot as for ES cells, or analyzed by PCR amplification with two sets of primers. Primers for the wt allele were derived from the cDNA sequence of mouse *ksr* CA1 domain: upstream primer, 5'-TATCTCCATCGGCAGTCT-3' (SEQ ID NO:20), downstream primer, 5'- TCGACGCTCACACT TCAA-3' (SEQ ID NO:21). The primers for the mutant allele were from the sequence of the neomycin phosphotransferase gene: upstream primer, 5'-CTGACCGCTTCCTCGTG-3' (SEQ ID NO:22); downstream primer, 5'-ATAGAGCCCACCGCATCC-3'(SEQ ID NO:23). The size of the expected product is 493-bp for the wt and 312-bp for the disrupted allele. Standard PCR conditions were employed: initial denaturation of 5 min at 94°C, followed by 30 cycles with annealing at 56°C, extension at 72°C, and denaturation at 94°C, all for 30 sec.

[0172] Northern and western blot analysis. Poly A^+ RNA was prepared from adult mouse tissues using the Oligotex kit from Qiagen Inc. (Valencia, CA). The blots were hybridized with a specific ^{32}P-labeled probe corresponding to the CA2-CA4 domains of murine *ksr* cDNA (1.47-kb). For embryonic tissues, we used a Mouse Embryo MTN Blot (BD Biosciences, San Diego, CA). Protein homogenates were prepared from $ksr^{+/+}$ and $ksr^{-/-}$ tissues, or MEFs in RIPA buffer and fractionated by SDS-PAGE (100 μg protein/lane). KSR expression was detected by western blot with a mouse monoclonal anti-KSR antibody (BD Biosciences, San Diego, CA) or a goat polyclonal anti-KSR antibody generated to amino acids 855 to 871 of KSR (c-19, Santa Cruz Biotechnology, Santa Cruz, CA). MEK and MAPK activation in MEFs were detected by western blot with anti-phospho-MEK and anti-phospho-MAPK specific antibodies: polyclonal anti-MEK, polyclonal anti-p44/42 MAPK, monoclonal anti-phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) and polyclonal anti-phosph-MEK1/2 (Ser^{217}/Ser^{221}) (Cell Signaling, Beverly, CA).

[0173] Histology. Skin tissues were collected from 10-day old $ksr^{+/+}$, $ksr^{-/-}$ and $egfr^{-/-}$ (kindly provided by Dr. Laura Hansen) mice and fixed for 15-18 hours in 10% neutral buffered formalin, washed 2 hours in 70% ethanol and embedded in paraffin blocks. The blocks were sectioned 4-6 μm thick, placed on glass slides and stained with hematoxylin and eosin.

[0174] MEF studies. MEFs, derived from $ksr^{+/+}$ and $ksr^{-/-}$ day 12-13 embryos, were prepared as described[15]. 0.25 x 10^6 early passage MEFs (PDL<6) were seeded in 6-well plates and grown in DMEM supplemented with 10% FBS for 24 h at 37°C. After 48 h in serum-free medium, cells were stimulated with 0.05-1.0 ng/ml EGF for 3 min, washed with PBS and lysed in 0.2 ml of NP-40 lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 2 mM EDTA, 10% Glycerol, 1% Nonidet P-40 plus protease and phosphatase inhibitors). Raf-1 activity was performed as previously described (27). Briefly, 300 ug of total lysate was immunoprecipitated with a polyclonal anti-Raf-1 antibody (Upstate Biotechnology, Lake Placid, NY), washed with NP-40 buffer containing 0.5M NaCl and incubated with the kinase-dead GST-MEK-1 (K97M). Activated MEK-1 was visualized by Western blot with a polyclonal anti-phospho-MEK antibody (Cell Signaling, Beverly, CA). To analyze cell proliferation, 0.15 x 10^6 *ksr*$^{+/+}$ or *ksr*$^{-/-}$ low-passage MEFs were seeded on 60 mm plates and counted at the indicated time points by hemacytometer. Data (mean+/-SD) are compiled from three independent experiments. To assess transformation capacity, MEFs from *ksr*$^{+/+}$ and *ksr*$^{-/-}$ mice were transduced sequentially with retroviral plasmids pWZL-Hygro-c-myc and pBabe-Puro-H-RasV12 (kindly provided by Scott Lowe, Cold Spring Harbor Laboratories), resuspended in 0.3% noble agar and seeded in 60 mm plates as described (15). Colonies consisting of at least 50 cells were counted after 3 weeks.

[0175] Generation of Tg.AC/*ksr*$^{-/-}$ mice. Homozygous male and female Tg.AC transgenic mice (16) were obtained at 3-4 week of age from Charles River Laboratories Inc. (Wilmington, MA). To produce the target population, *ksr*$^{-/-}$ mice were first bred to hemizygous Tg.AC mice containing the *v*-Ha-*ras* transgene. The resulting F1 females and males, heterozygous for *ksr* and hemizygous for the Tg.AC transgene, were then bred to obtain offspring in the *ksr* background. Nonresponder Tg.AC mice (17) were excluded from the study group. Presence of the Tg.AC transgene was determined by PCR amplification as follows: initial denaturation of 1 min 10 sec at 74°C, followed by 30 cycles with annealing at 55°C for 1min, extension at 72°C for 3min, and denaturation at 94°C for 1 min. The sequence of the Forward Primer was 5'-GGAACCTTACTTCTGTGGTGTGAC-3' (SEQ ID NO:13), and the sequence of the Reverse Primer was 5'-TAGCAGACACTCTATGCCTGTGTG-3' (SEQ ID NO: 14). PCR results were confirmed by Southern blot analysis as described (17).

[0176] Skin tumor experiments. Mice were treated twice weekly with 5 μg TPA (Sigma Chemical Company, St. Louis, Missouri) for 15 weeks and observed for papilloma development as described (16). Offspring from the original Tg.AC mice in the FVB/N background from Charles River Laboratory were used as controls. Papillomas were counted weekly for 20 weeks. *v*-Ha-*ras* transgene expression in skin after TPA treatment was assessed by nested PCR as described (24).

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EXAMPLE 2

ABSTRACT

[0177] Given the prevalence of oncogenic *ras* mutations in human cancers, selective inactivation of gain-of-function *(gf)* Ras signaling represents a highly attractive therapeutic approach, although it has not been achieved clinically. Here, *gf* Ras signaling was targeted indirectly via genetic or pharmacologic inactivation of Kinase Suppressor of Ras1 (KSR1), an immediate downstream effector selective for *gf* Ras. KSR1 inactivation abrogated *gf* Ras-mediated tumorigenesis induced by constitutively activated epidermal growth factor receptor or oncogenic K-Ras mutation in several human tumor cell lines and in nude mice xenografts. Inhibition of *ksr1* via continuous infusion of KSR antisense oligonucleotides (AS-ODNs) prevented growth of oncogenic K-*ras*-dependent human PANC-1 pancreatic and A549 non-small-cell lung carcinoma (NSCLC) xenografts in nude mice, effected regression of established PANC-1 tumors, and inhibited A549 lung metastases, without apparent toxicity. These studies suggest KSR AS-ODNs as a treatment for *gf* Ras-dependent human malignancies, in particular pancreatic cancer for which there is presently no effective curative therapy.

INTRODUCTION

[0178] Adenocarcinoma of the exocrine pancreas represents the fourth-leading cause of cancer-related mortality in Western countries. Treatment has had limited success and the five-year survival remains less than 5% with a mean survival of 4 months for patients with surgically unresectable tumors (1,2) . While oncogenic activation of H-, K-, and N-Ras arising from single nucleotide substitutions has been observed in 30% of human cancers (5) ,over 90% of human pancreatic cancer manifest the codon 12 K-*ras* mutation (3-5) . This point mutation can be identified early in the course of the disease when normal cuboidal pancreatic ductal epithelium progresses to a flat hyperplastic lesion, and is considered causative in the pathogenesis of pancreatic cancer (6,7) . The regulation of oncogenic K-*ras* signaling in human pancreatic cancer, however, remains largely unknown. While various therapeutic strategies have been developed to inactivate key components of the Ras-Raf-MAPK cascade, specific inhibition of *gf* Ras action has not been achieved clinically (8,9).

[0179] Recent studies demonstrated that Kinase Suppressor of Ras (KSR1) positively modulates the Ras/MAPK signaling arm of the EGFR/Ras pathway. KSR1 acts downstream of Ras, either upstream of or parallel to Raf in *Drosophila* and *C. elegans* (10-12) . Mammalian forms of KSR1, identified on the basis of sequence homology (12), suggest that KSR signaling is evolutionarily conserved. The precise mechanisms of mammalian KSR signaling and its biological functions, however, remain largely unknown. Genetic studies from *ks*r1-deficient *C. elegans* and mice demonstrate that while *ks*r1 is dispensable for normal development (10,11,13) , it may be obligate for *gf* Ras signaling through the MAPK cascade (see Example 1 above). In *C. elegan*s, KSR loss-of-function *(lf*) reverts the *gf* Ras-mediated multivulva phenotype (10,11) caused by the same codon 13 mutation that confers oncogenic potential onto mammalian Ras.

[0180] To elucidate the role of KSR1 in Ras-mediated human malignancies, especially in pancreatic cancer, and to explore the feasibility of employing KSR1 as a therapeutic target, antisense approaches were employed to genetically and pharmacologically inactivate mammalian *ks*r1. We report here that both approaches to KSR1 inactivation abrogated *gf* Ras signaling of tumorigenesis, either via constitutively activated EGFR or oncogenic K-Ras mutation. Further, antisense-mediated inhibition of *ks*r1 gene expression via continuous infusion of KSR AS-ODNs prevented the oncogenic K-*ra*s-dependent growth of human PANC-1 pancreatic and A549 non-small-cell lung carcinoma (NSCLC) xenografts in nude mice, elicited regression of established PANC-1 tumors, and inhibited A549 lung metastases without apparent toxicity. These studies demonstrate that KSR AS-ODNs might represent a tumor-specific therapeutic agent for the treatment of oncogenic K-*ras*-dependent human malignancies.

RESULTS

[0181] Inhibition of *ksr*1 gene expression induces morphologic changes in A431 cells. In *C. elegans*, KSR1 regulates *gf* Ras signaling of vulval development, a pathway initiated through LET-23, the EGFR homolog (10,11) . To explore the role of mammalian KSR1 in EGFR-mediated tumorigenesis, we employed the A431 human epidermoid carcinoma tumor line in which tumor growth is driven through wild type Ras by a 100-fold excess of activated EGFR/HER1 (10^7 receptors/cell) (14). We generated A431 cell lines stably expressing inducible forms of wild type KSR1 (KSR-S), antisense KSR1 (KSR-AS) and dominant negative KSR1 (DN-KSR) using the Retro-Tet-Off system. While Flag-tagged KSR-S and DN-KSR were expressed to similar levels (FIGURE 5A), stable expression of the KSR-AS resulted in a 60% reduction of endogenous KSR expression (FIGURE 5B). Further, doxycycline (Dox) treatment elicited a dose-dependent inhibition of KSR-S expression (FIGURE 5C), and Dox withdrawal following its addition effectively restored KSR-S expression (not shown). Similar results were found in the DN-KSR and KSR-AS cells (not shown). These observations indicate that the KSR-Tet-Off system is tightly regulated by Dox.

[0182] The effect of manipulating KSR1 levels on the morphology of stably transfected A431 cells was examined first. While non-transfected (not shown), vector-transfected and KSR-S-transfected A431 cells displayed the similar cobblestone morphology of poorly differentiated squamous epithelial cells (FIGURE 5D), abrogation of *ksr*1 expression by KSR-AS produced a marked change in cell morphology. The KSR-AS cell somata gradually enlarged and flattened, cytoplasmic processes retracted, and cells grew in a more scattered pattern (FIGURE 5D). Further, these cells became multinucleated (FIGURE 5E), indicative of the failure to complete cytokinesis with a resultant proliferation defect (see below) (15). Phase-contrast microscopy reveals that while over 80% of KSR-AS cells contained multiple nuclei, multinucleated cells were rarely seen (<8%) in control or KSR-S cells. Similar morphologic changes were observed in DN-KSR cells (FIGURE 5D and 5E) indicating that inhibition of *ksr*1 gene expression in A431 cells might have profound effects on EGFR-mediated biological events in this tumor cell line.

[0183] Inhibition of *ksr*1 gene expression attenuates A431 tumorigenesis. To assess the consequences of KSR1 inhibition on the malignant properties of A431 cells and their response to EGF *in vitro*, cell proliferation, invasion and transformation assays were performed. When KSR-S was overexpressed by A431 cells, both baseline and EGF-stimulated proliferation (FIGURE 6A), invasion (FIGURE 6C) and transformation (FIGURE 6D) were markedly enhanced. In contrast, depletion of *ksr*1 expression in KSR-AS cells resulted a significant inhibition of baseline proliferation, invasion and transformation (FIGURE 6, $p<0.05$ in each case), and the abrogation of EGF responses (FIGURE 6). The DN-KSR effect was similar to that observed with KSR-AS (FIGURE 6).

[0184] Consistent with the observed alterations in cell growth, KSR significantly impacted cell cycle distribution as determined by FACS analysis (FIGURE 6B). While there was a significant elevation of S-phase cells in exponentially growing KSR-S cells, a sharp reduction in S-phase cells coupled with a concomitant increase of G2/M-phase cells was observed in KSR-AS cells compared to vector-transfected controls ($p<0.05$ in each case). These observations were confirmed by Ki-67 staining (not shown). The specificity of KSR-S and KSR-AS in mediating stimulation and inhibition, respectively, of proliferation, invasion and transformation was confirmed by turning off the KSR-S and

KSR-AS expression by Dox treatment (not shown). These observations demonstrate that while overexpression of KSR enhanced the neoplastic properties of A431 cells, inactivation of KSR by KSR-AS or DN-KSR rendered A431 cells less malignant.

[0185] To elucidate whether KSR1 down-regulation might have a similar anti-proliferative effect *in vivo*, 10^6 KSR-S, KSR-AS, DN-KSR or vector-transfected A431 cells were injected subcutaneously (s.c.) into the right flank of immunodeficient (nude) mice. While tumor take was 100% in mice receiving KSR-S and vector-transfected cells, KSR-S tumors had an earlier onset (FIGURE 7A, left-shifted growth curve, $p<0.05$), were 200% larger in size on day 25, and had 2.5-fold more Ki-67 positive cells than vector-transfected tumors of the comparable size (not shown). Examination of tumor specimens removed on day 25 revealed continued expression of Flag-KSR-S (not shown). The specificity of KSR-S in mediating these effects was confirmed by feeding a group of KSR-S tumor-bearing mice with Dox-containing water which shut off tumoral KSR-S expression efficiently (not shown), and almost completely prevented the growth stimulatory effect of KSR-S on A431 tumors (FIGURE 7A, KSR-S *vs.* KSR-S + Dox, $p<0.01$). In contrast, mice injected with 10^6 A431 KSR-AS or DN-KSR cells failed to develop any tumors when observed up to 120 days (FIGURE 7A and not shown). When the inocula size was increased to 10×10^6 and prepared in 50% Matrigel, only 1 out of 20 mice in each case developed a late onset (day 42 for KSR-AS and day 36 for DN-KSR) slow growing tumor. Further, squamous differentiation was evident in both the vector- and KSR-S tumors (FIGURE 7B *(i)* and *(ii)*, black arrows), although KSR-S tumors had less kertohyalin granules and a higher mitotic index (not shown). In contrast, squamous differentiation was absent from KSR-AS and DN-KSR tumors (FIGURE 7B *(iii)* and *(iv)*). Moreover, consistent with our observations *in vitro*, 25% of the KSR-AS and 18% of the DN-KSR tumor cells were multinucleated *in vivo* (FIGURE 7B *(iii)* and *(iv)*, black arrows, and *(v)*).

[0186] These observations demonstrate that inhibition of A431 tumorigenesis by KSR-AS involves attenuation of proliferation and induction of multinucleation.
To confirm that prevention of A431 tumorigenesis by KSR-AS is due to inhibition of *ksr*1 expression by KSR-AS, we designed phosphorothioate AS-ODNs against the unique CA1 domain (SEQ ID NO: 1) (amino acids 42-82 (SEQ ID NO:2)) of KSR1, which is conserved between the mouse and human (12), to inactivate *ksr*1 expression pharmacologically. Among the AS-ODNs tested, the AS-ODN against nucleotides 214 to 231 (SEQ ID NO:5) of KSR1 (designated AS-ODN1(214-231)), which has no sequence homology to any other mammalian gene, exhibited the most potent and specific antisense effect, and was chosen for further characterization. *In vitro* treatment of A431 cells with 1 μM KSR AS-ODN (SEQ ID NO: 8) for 24 h resulted in a 90% reduction of endogenous KSR1 expression as determined by immunofluorescence staining (FIGURE 8A) and Western blotting (not shown), while control ODNs had no apparent effect (FIGURE 8A and not shown). Moreover, expression of other cellular proteins including the EGFR, H-Ras, c-Raf-1 and MAPK was not altered by treatment with KSR AS-ODN or control ODNs (not shown), indicating that the antisense effect was specific for KSR. Similar to inactivation of KSR by stable expression of full-length KSR-AS, KSR AS-ODN treatment attenuated A431 cell proliferation (FIGURE 8B) and invasion (FIGURE 8C) in a dose-dependent fashion ($p<0.05$). At 1 mM, KSR AS-ODN inhibited A431 cell proliferation and invasion by 80% and 70%, respectively. In contrast, Control-ODN (FIGURE 8C), which lacks homology to any mammalian gene (16), or Sense-ODN or mismatch AS-ODNs (not shown), were ineffective.

[0187] To assess the antitumor activity of KSR AS-ODNs *in vivo*, AS-ODNs or control ODNs were delivered via continuous s.c. infusion to provide sustained tumor exposure. Infusion was initiated two days prior to tumor implantation in order to reach a steady state ODN plasma level. 10^6 A431 cells were injected s. c. into nude mice to obtain seed tumors of 400 mm^3. Approximately 50 mg of the freshly prepared seed tumor fragments were then transplanted to AS-ODN- or Control ODN-treated mice. Treatment with KSR AS-ODN at a low dose of 5 mg/kg/day effectively reduced tumoral KSR1 levels by 85% and attenuated A431 tumor growth by 80% (FIGURE 8D, $p<0.01$), without apparent toxicity, consistent with the known lack of toxicity of this therapeutic approach (17). In contrast, no antitumor effects were observed following treatment with vehicle alone (saline) or with identical doses of the Control-ODN, or Sense-ODN (FIGURE 8D and not shown). Similar results were obtained when treatment was initiated using mice with established A431 tumors of 150 mm^3 (not shown). Collectively, these results demonstrate that KSR1 is obligate for EGFR signaling of A431 tumorigenesis *in vivo* via hyperactivated wild type Ras. Further, the antitumor activity of KSR AS-ODNs appeared to be achieved via selective inhibition of *ksr*1 gene expression with high specificity. These studies suggest that it might be feasible to use KSR AS-ODN to abrogate EGFR/Ras signaling of human tumorigenesis.

[0188] <u>Inhibition of *ksr*1 expression abrogates oncogenic K-*ras*-mediated human pancreatic tumorigenesis via specific attenuation of Ras/Raf-MAPK signaling.</u> To elucidate the importance of KSR1 in mediating oncogenic Ras signaling of human tumorigenesis and to explore the therapeutic potential of KSR AS-ODNs, we employed the human pancreatic cancer PANC-1 xenograft mouse model. This tumor manifests the oncogenic codon 12-mutation of K-*ras*. Similar to A431 cells, treatment of PANC-1 cells *in vitro* with KSR AS-ODNs attenuated cell proliferation (FIGURE 9A), invasion (FIGURE 9C) and transformation (FIGURE 9D) in a dose-dependent fashion (FIGURE 9 and not shown) ($p<0.05$ in each case). Further, treatment with 5 μM AS-ODN led to a 90% reduction of endogenous KSR1 expression (FIGURE 9E). To confirm the effectiveness of KSR AS-ODN in inhibiting oncogenic K-*ras* function, a panel of codon-12 K-*ras* mutated human pancreatic cancer cell lines were treated with 5 μM of KSR AS-ODN and assayed for cell proliferation. While cell growth was inhibited by 50 to 80% in all cell lines after AS-ODN treatment ($p<0.01$ each), Sense-ODN had no apparent effect (FIGURE 9B).

[0189] We previously demonstrated that KSR1 activation is required for c-Raf-1 and subsequent MAPK activation *in vitro* in response to mitogenic doses of EGF stimulation (18,19). To molecularly order KSR1 and Raf-1 in oncogenic K-*ras* signaling, PANC-1 cells were treated with 5 μM AS-ODN, transfected with the dominant positive BXB-Raf-1 and assayed for cell invasion and transformation. If Raf-1 is downstream of KSR, *gf* Raf-1 (BXB-Raf-1) should reverse the inhibitory effect of KSR inactivation by AS-ODNs on PANC-1 cell invasion and transformation. Indeed, while BXB-Raf-1 had no effect on endogenous KSR1 expression (FIGURE 9E), it completely reversed the inhibitory effect of AS-ODN on PANC-1 cell invasion (FIGURE 9C) and transformation (FIGURE 9D). These observations indicate that c-Raf-1 is epistatic to KSR1, consistent with our *in vitro* findings and with the current literature (19-22). Additional studies were performed to examine the mechanism by which KSR1 inactivation affected oncogenic Ras-mediated intracellular signaling. For these studies, AS-ODN-treated and BXB-Raf-1-transfected PANC-1 cells were serum-depleted for 48 h and stimulated with 1 ng/ml of EGF. MAPK and PI-3 kinase activation were assayed by Western blot analysis using phospho-MAPK and phospho-Akt specific antibodies. While AS-ODN treatment blocked EGF-induced MAPK activation (FIGURE 9F, upper panel, lane 6 *vs.* lane 2), it had no apparent effect on Akt activation (FIGURE 9F, lower panel, lane 6 *vs.* lane 2). Sense-ODN had no effect on either MAPK or Akt activation (FIGURE 9F). Moreover, the inhibitory effect of AS-ODN on MAPK activation could be completely reversed by expression of BXB-Raf-1 (FIGURE 9F, upper panel, lane 4 vs. lane 2). Total MAPK and Akt content were largely unaffected by treatment with ODNs or transfection with BXB-Raf-1 (FIGURE 9F and not shown). These results suggest that abrogation of oncogenic K-Ras signaling in pancreatic cancer cells by KSR AS-ODN is likely achieved by specific inhibition of the Ras-Raf-MAPK cascade. To test the therapeutic potential of

KSR AS-ODNs to treat human pancreatic cancer, PANC-1 xenografts either derived from 10^6 cultured PANC-1 cells (FIGURE 10A *(i)*), or from freshly harvested seed PANC-1 tumors (prepared as described above) (FIGURE 10A *(ii)*), were transplanted into nude mice. The steady state plasma AS-ODN levels for the 5 and 10 mg/kg/day doses of infusion were determined by OliGreen and HPLC assays to be 63 and 123 ng/ml, respectively, consistent with that reported in the literature using similar doses (23). For PANC-1 tumors arising from the injected cells, tumors were allowed to reach 100 mm^3 prior to the initiation of AS-ODN treatment. Infusion of AS-ODNs at 10 mg/kg/day for 14 days resulted in 40% reduction in tumor volume with a 100% response rate (FIGURE 10A *(i)*, $p<0.05$ vs. Control-ODN). A group of AS-ODN treated tumors that had regressed were monitored for tumor re-growth after the treatment was discontinued. Only 1 of 5 tumors exhibited re-growth while the rest remained regressed and stable for up to 4 weeks (not shown). For PANC-1 xenografts propagated via serial passage *in vivo,* continuous infusion of KSR AS-ODNs, initiated 2 days prior to tumor transplantation, attenuated the growth of PANC-1 tumors in a dose-dependent fashion (FIGURE 10A *(ii)*). No apparent toxicity (weight loss, behavioral alteration, organomegaly, inflammation, bleeding) was observed at any dose and was confirmed by histologic examination of numerous tissues at autopsy (not shown). At 75 mg/kg/day, PANC-1 tumor growth was completely abolished and all mice remain tumor-free up to 4 weeks after the treatment was discontinued (FIGURE 10A *(ii)* and not shown). In contrast, treatment with vehicle alone (saline), Control-ODN, or Sense-ODN exhibited no antitumor effects at all doses examined (FIGURE 10A and not shown). These observations support the conclusion that the antitumor effects observed for KSR AS-ODNs occur through an antisense mechanism of action. Similar anti-neoplastic effects of KSR AS-ODN were observed in PANC-1 tumors transplanted orthotopically under the pancreatic capsular tissue (not shown).

[0190] To confirm the specificity of KSR in mediating K-*ras* signaling of pancreatic tumorigenesis, we examined endogenous *ksr*1 gene expression in saline-, Sense-ODN and AS-ODN-infused PANC-1 tumors. KSR1 expression was inhibited by 90% in all AS-ODN-treated animals examined, while it was largely unchanged by saline or Sense-ODN infusion (FIGURE 10B), confirming a sequence-specific target effect. As an additional control, the effect of AS-ODN treatment on Ras activation *in vivo* was measured by determining the amount of GTP-Ras in PANC-1 tumors using the GST-RBD-Raf-1 pull down assay as described in Methods. Consistent with the data of A431 and PANC-1 cells in culture (not shown), AS-ODN treatment had no apparent effect on Ras activation (FIGURE 10C), indicating that signaling events upstream of Ras activation were intact and inactivation of oncogenic K-*ras* signaling in PANC-1 cells by KSR depletion occurs downstream of Ras.

[0191] To confirm the effectiveness of KSR AS-ODNs in hindering other oncogenic K-*ras*-dependent human tumors, the codon 12 K-*ras* mutated A549 human non-small-cell lung carcinoma model (NSCLC) was selected. For these studies, 50 mg A549 seed tumor fragments, prepared similarly to A431 and PANC-1 seed tumors as above, were transplanted s. c. into nude mice. Treatment with KSR AS-ODN was initiated when A549 tumors reached 150 mm^3 . At 10 mg/kg/day, KSR AS-ODN completely inhibited the growth of the established A549 tumors while Saline, Control-ODN or Sense-ODN had no apparent effect (FIGURE 10D *(i)*). When animals were sacrificed at the end of the experiment, lungs were resected from Sense-ODN- or AS-ODN-treated mice and stained with Indian ink to visualize surface lung metastases derived via systemic dissemination. Control-ODN-treated lungs had an average of 8-11 metastatic foci. AS-ODN treatment elicited a dose-dependent inhibition of A549 lung metastasis (FIGURE 10D *(ii)*, $p<0.05$). These observations suggest that while KSR1 is obligate for K-*ras*-dependent primary tumor growth, it may also play an essential role in the metastatic progression of these tumors. Further, KSR AS-ODN could be an effective agent in the management of K-*ras*-dependent human malignancies.

DISCUSSION

[0192] The present studies provide evidence that KSR1 is obligate for *gf* Ras signaling at he tissue level, and that inhibition of *ksr1* expression leads to selective regression of *gf* Ras-dependent tumors. Previous clinical studies designed to treat *gf* Ras-dependent tumors by inhibition of elements of the Ras/Raf-1/MAPK signaling cascade have to date been largely unsuccessful (9). While toxicity for most agents has been acceptable, success of treatment has been limited by lack of specificity in inhibiting different Ras isoforms, which recent data suggest may have distinct biologic functions (24-26) and lack of selectivity towards *gf* versus physiologic Ras signaling (8,9). Similar problems exists for experimental drugs designed to inhibit elements of the Raf-1/MEK1/MAPK cascade (9). The present studies on the effects of KSR AS-ODNs provide an approach to specifically attenuate *gf* Ras signaling in the treatment of Ras-dependent human tumors.

[0193] While the targeting of DNA sequences with AS-ODN technology represents an attractive therapeutic approach to the treatment of cancer (27), a principle problem with this approach has been the designation of specificity of the AS-ODN effect for the gene of interest. We provide a number of different lines of evidence to support the notion that the inhibition of tumorigenesis observed with KSR AS-ODNs is due to selective inactivation of *gf* K-*ras* signaling via inhibition of *ks*r1. Our data show that genetic inhibition of *ks*r1 expression by the KSR-AS Tet-Off construct yielded comparable antisense-mediated effects *in vitro* and *in vivo* as KSR AS-ODNs. Further, various ODNs, designed to control for sequence-dependent and sequence-independent non-antisense artifacts, had no effects on *ks*r1 gene expression, or on tumor growth in cell culture or *in vivo*. In addition to ODN sequence specificity, the effects of KSR AS-ODN on *ks*r1 expression were specific for the intended target as expression of other genes of the EGFR-Ras-MAPK pathway was unaffected. Finally, conditional overexpression of

KSR-S by A431 cells delivered a phenotype opposite to KSR inactivation by KSR-AS, and both KSR-S and KSR-AS effects were reversible in the Tet-Off system by turning off expression by Dox treatment. These results collectively attest that the antitumor effects of KSR AS-ODN are achieved by an antisense mechanism.

[0194] The lack of normal tissue toxicity in animals treated with KSR AS-ODNs is consistent with recent reports that *ksr*1 is dispensable for normal development in *C. elegans* and mice (10,11,13, and see Example 1 above). As recent investigations have uncovered a second *ksr* allele, *ksr*2 in *C. elegans* (28) and in mice (29), the lack of tissue toxicity after depletion of *ksr*1 by KSR AS-ODN might be due to compensation by *ksr*2 for normal cellular functions. Alternately, the lack of toxicity may reflect topological distribution of KSR. Recent evidence suggests that elements of the Ras/Raf-1/MAPK pathway are compartmentalized in more than one type of membrane microdomains (26,30), and that compartmentalization is associated with regulation of activity. In this regard, Ras and c-Raf-1 associate with sphingolipid enriched microdomains (also known as rafts) in the plasma membrane and with the bulk membrane fraction. Raft association, at least for c-Raf-1, appears to involve binding to the sphingolipid ceramide (31). Further, depending on activation status, Ras forms may traffic between compartments (32), with *gf* Ras preferentially targeting the bulk membrane.

[0195] Whether KSR, which some groups argue is ceramide-activated (20,33), plays a specific role in the *gf* Ras activation process, and hence its inactivation would marginally affect normal cellular function, will be the topic of future investigation. Lastly, the apparent lack of toxicity of our KSR ODN is not surprising as the phosphorothioate class of AS-ODNs, the most commonly used AS-ODNs, are generally well tolerated. Administration via continuous infusion in preclinical models and in human clinical trials have established that sequence-independent toxicities (activation of the complement system, prolongation of activated partial thromboplastin time and alterations of hematological parameters) are usually not encountered at doses at which pharmacologic antisense effects are achieved (34-36).

[0196] These studies also suggest that the therapeutic benefit of KSR AS-ODNs may not be limited to oncogenic K-*ras*-dependent human cancers, but might include a broader spectrum of tumor types, as our studies with KSR AS-ODNs were found effective against the tumor line A431, which is driven by hyperactivated wild type Ras. The therapeutic action of KSR AS-ODNs on established tumors *in vivo* likely involves both inhibition of tumor cell proliferation and induction of tumoral cell death. The anti-proliferative effect of KSR1 inactivation was evident by a decrease in cells in S phase and the induction of multinuclei phenotype *in vitro* and *in vivo*. Additionally, AS-ODN-treated A431 and PANC-1 tumors contained large necrotic areas (60-80% of the surface of the cut section). The mechanism of the latter effect, however, remains unknown. Previous studies demonstrated that significant microvascular endothelial apoptosis might also contribute to the anti-tumor effect as a result of *ras* inactivation (37). However, in our models, only sporadic endothelial cell apoptosis was detected by CD34 and TUNEL staining in PANC-1 tumors treated with KSR AS-ODNs (not shown). The role of KSR in angiogenesis must await further investigation in more relevant models of tumor angiogenesis.

[0197] Another important finding emerging from the present study is that KSR appears required for oncogenic Ras-mediated tumor metastatic progression. Inhibition of A549 lung metastases with KSR AS-ODN treatment is in agreement with our preliminary data that MMP-2 and 9 activities were increased in A431-KSR-S cells and inhibited in A431-KSR-AS cells (data not shown). Investigations are underway to elucidate the role of KSR in tumor progression.

[0198] The effective use of KSR AS-ODNs also provides the potential for improved understanding of the regulation of critical downstream events involved in *gf* Ras signaling, which at the present time, are only partially known. Raf-MAPK and PI-3 kinase modules are two established downstream pathways mediating *gf* Ras signaling of tumorigenesis (38-41). Here we provided evidence that KSR1 functions as a critical mediator of *gf* Ras likely via specific regulation of the Raf-1-MAPK signaling arm. Support for this notion is derived from recent studies demonstrating that MMTV-MT-dependent mammary tumor genesis, signaled primarily via src and PI-3 kinase via wild type Ras, was not affected in *ksr* -/- mice (13). In contrast, tumor genesis of oncogenic v-Ha-Ras-mediated epidermal skin tumors, signaled through the c-Raf-1/MAPK cascade, was abrogated in *ksr1* -/- mice (Lozano and Kolesnick, unpublished). Further, the present studies with KSR antisense support the molecular ordering of c-Raf-1 as epistatic to mammalian KSR1, which is consistent with genetic results from *Drosophila* and *C. elegans* (10,11). We believe these observations may help to resolve some of the disputes regarding upstream elements of the Ras/Raf-1/MAPK module.

[0199] In summary, the current study provides original observations supporting KSR1 as a new molecular target for the treatment of human malignancies dependent on *gf* Ras signaling.

METHODS

[0200] Cell culture and generation of Retro-Tet-Off A431 cell lines. Human epidermal carcinoma cell line A431, lung carcinoma cell line A549 and pancreatic cell lines PANC-1, Capan-2, PL-45, HPAF-II, AsPc-1 and MiapaPa-2 were obtained from ATCC (Manassas, VA). The full-length wild type mouse *ksr*1 cDNA, which is over 90% identical to human *ksr*1 (12), was cloned in both sense (KSR-S) and antisense (KSR-AS) orientations into the pRetor-TRE under a doxycycline-inducible promoter in pRetro-Tet-Off (Clontech, Palo Alto, CA). DN-KSR (D683A/D700A/R589M) was sub-cloned similarly. A431 cells were infected with medium collected from PT67 packaging cells transfected with KSR-S, KSR-AS, DN-KSR or the empty vector, and maintained under double selection (0.1 mg/ml neomycin and 0.1 mg/ml Hygromycin).

[0201] Western blot, immunofluorescence and immunohistochemistry. Total cell lysates and tumor lysates were prepared in NP-40 buffer as described (18,42). Immunoprecipitation (IP) or Western blotting (WB) was performed according to the manufacturer's protocols with the following antibodies: monoclonal anti-Flag M2 antibody from Sigma (St Louis, MO), polyclonal anti-p44/42 MAPK, monoclonal anti-phospho-p44/42 MAPK (Thr202/Tyr204), polyclonal anti-phospho-MEK1/2 (Ser217 /Ser221) and polyclonal anti-phospho-Akt (Ser 473) antibodies from Cell Signaling (Beverly, CA), and polyclonal anti-c-Raf-1 antibody from Upstate Biotechnology Inc. (Lake Placid, NY). Endogenous KSR1 expression was determined by immunoprecipitation and WB analysis from 1 mg of total lysates or by immunofluorescence microscopy, using the monoclonal anti-KSR antibody (BD Biosciences, San Diego, CA) (1:100 dilution) and HRP- or Texas-Red-conjugated goat anti-mouse secondary antibodies, respectively (Molecular Probes, Eugene, Oregon). Histology and immunohistochemistry were performed on formalin-fixed, paraffin-embedded tumor or tissue specimens. 5 mm-cut sections were deparaffinized, rehydrated in graded alcohols, and H & E stained or immunostained using the avidin-biotin immunoperoxidase (Vector Laboratories, Burlingame, CA) method (43). The following primary antibodies were used: rat anti-mouse CD34 (1:50) antibody from PharMingen (San Diego, CA) and polyclonal anti-human Ki67 antibody (1:100) (Vector Laboratories). Diaminobenzidine was used as the chromogen and hematoxylin as the nuclear counterstain as described (43). Apoptosis were assessed by terminal deoxy transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) (Roch, Mannheim, Germany) as described (43).

[0202] Proliferation, Matrigel invasion and soft agar transformation assays. 2 x 10^4 A431 cells or 1-3 x 10^4 human pancreatic cells were plated in 6-well plates. The total number of cells/well was counted at the indicated time points to construct cell growth curves. For EGF treatment, 1.0 ng/ml of EGF was added to the culture and replaced every other day. The invasion assay was performed as described (42). Cells on the underside of the filters were counted in 10 randomly chosen fields (40X magnification) and reported as an average number of cells invaded per field. For EGF treatment, cells were replaced with serum-free medium for 2 h prior to the experiment. The Soft Agar assay was set up in 35mm culture plates coated with 1.5 ml culture medium containing 0.5% agar and 5% FBS. 5 x 10^3 cells were suspended in 1.5 ml medium containing 0.1% agar and 5% FBS, and added to the agar pre-coated plates. Colonies consisting of more than 50 cells were scored after 14-21 days of incubation using a dissecting microscope. For EGF treatment, FBS was omitted from the culture medium.

[0203] Cell cycle analysis. Cell cycle distribution was determined by FACS analysis. For these studies, cell pellets collected from exponentially growing monolayers were washed twice with PBS containing 0.5% FBS and fixed with 100% ethanol for 15 min. Fixed cells were treated with RNase A (0.1 mg/ml) for 30 min at 37°C and stained with propidium iodide (0.05 mg/ml). The proportion of cells in the different phases of the cell cycle was calculated from the experimental fluorescence histograms.

[0204] *In vitro* treatment with KSR AS-ODN. KSR1 AS-ODN (5'-CTTTGCCTCTAGGGTCCG-3') (SEQ ID NO: 8)(AS-ODN1(214-231)) and KSR sense-ODN (5'-CGGACCCTAGAGGCAAAG-3') (SEQ ID NO: 15) were generated as phosphorothioate derivatives against nucleotides 214 to 231 (SEQ ID NO: 1) of the unique CA1 domain (amino acids (AAs) 42-82) of KSR1 by Genelink Inc. (Hawthorne, NY). Control ODN (5'-CACGTCACGCGCGCACTATT-3') (SEQ ID NO: 16) was prepared similarly. For *in vitro* studies, ODNs were dissolved in sterile water and delivered to cells by Oligofectamine (Invitrogen, Carlsbad, CA) when cells were 30-40% confluent according to manufacturer's instructions. Cell proliferation was assayed at the indicated time points. 48 h after treatment, invasion and transformation assays were set up as above. For some studies, Control- and AS-ODN-treated PANC-1 cells were transfected with the dominant positive RSV-Raf-BXB (kindly provided by Dr. Joseph Bruder, NCI).

[0205] Tumor induction and *in vivo* treatment with KSR AS-ODN. For tumor induction, 10^6 cultured tumor cells suspended in 0.1 ml of PBS, or 50 mg of tumor fragments freshly harvested from serial passaged seed tumors, were transplanted subcutaneously into the right lateral flank of 6-8 wk old male athymic NCRnu (Germantown, NY). Tumor growth was measured every other day by calipers and tumor volume calculated as described (42). To determine the specificity of KSR-S on A431 tumorigenesis, a group of KSR-S tumor-bearing mice were fed with Dox-containing water (100 mg/ml). To determine the antitumor activity of KSR-AS ODN *in vivo*, infusion with Sense-, Control- or AS-ODNs via Alzet osmotic minipumps was initiated either 2 days prior to tumor transplantation or when tumor reached 100-150 mm^3 . A 5.0 - 75 mg/kg body weight/day dose range of ODN was chosen based on similar AS studies *in vivo* (34,44)

[0206] Ras activation assay. Ras activation status (GTP-Ras) in control ODNs or AS-ODN-treated PANC-1 cells or tumors was measured using the Ras activation assay kit (Upstate Biotechnology Inc., Lake Placid, NY) according to manufacturer's instructions as described (45).

[0207] Statistical analysis. All data were evaluated by the Student's *t* test (two-tailed) with $p < 0.05$ considered significant.

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EXAMPLE 3

[0208] Additional antisense oligonucleotides were synthesized and tested by proliferation assay in A431 cells as indicated in TABLE 1. Antisense nucleotides and their sequences were selected on the basis of criteria (based on hybridization conditions) to have no stable homodimer formation, no hairpin loop formation , no self-complementary sequences, and to have no stable duplex formation (<-6 kcal/mol. Sequences were selected using the Oligo 4 program (Molecular Biology Insights, Inc., Cascade, CO) and subsequently verified with the Oligo Tech program (Oligo Therapeutics Inc., Wilsonville, OR). Antisense oligonucleotides were generated against nucleotides 151 to 168 (AS-ODN3(151-168) (AS oligo sequence 5'-CAGCCCGCGCAGACTGCC-3') (SEQ ID NO: 6) and nucleotides 181 to 198 (AS-ODN2(181-198)) (AS oligo sequence 5'-GAGGTCGTTAGACACTGA-3') (SEQ ID NO: 7) of the KSR CA1 domain (both were P-S oligonucleotides). These oligonucleotides were tested along with the AS-ODN oligonucleotide against nucleotides 214 to 231 (AS-ODN1(214-231)) (AS oligo sequence SEQ ID NO: 8) described in Example 2. These antisense oligonucleotides represent reverse complements of nucleic acids encoding the amino acid sequences GSLRGL (SEQ ID NO: 17), AVSNDL (SEQ ID NO: 18) and RTLEAK (SEQ ID NO: 19) of the CA1 domain of KSR, respectively. A431 cells were transfected with the indicated amount of KSR AS-ODNs and cell proliferation was assessed 72 hr after the treatment. The effect of AS-ODN on A431 cell proliferation was presented as percent of inhibition of vehicle-treated (Oligofectamine alone) controls. This is a representation of one of four similar studies.

TABLE 1.Screening of KSR AS-ODNs by proliferation assay in A431 cells

Concentration of ODN (nM)	AS151-168 (% inhibition)	AS181-198 (% inhibition)	AS214-231 (% inhibition)	Control ODN (% inhibition)
10	0	0	8	2
50	0	5	20	3

100	3	15	25	2
200	16	23	42	0
400	24	42	67	4
800	38	58	87	3

EXAMPLE 4

[0209] To establish the specificity of KSR1 in mediating *gf* Ras signaling, the well-characterized human chronic myeloid leukemia cell line K562 was employed. K562 is Bcr-abl-driven and is therefore independent of *gf* Ras signaling. The specific and dose-dependent inhibition of PANC-1 cell proliferation by AS-ODN treatment is depicted in FIGURE 13. PANC-1 and K562 cells were treated with the indicated doses of Control- or AS-ODNs and cell proliferation assays were performed (FIGURE 13A). AS-ODN-1 and AS-ODN-2 correspond to nucleotides 214-231 and 181-198 of *ksr1* cDNA, respectively. While PANC-1 cell proliferation was inhibited as expected, K562 cell proliferation was unaffected by ODN treatment. Treatment of K562 cells with 5 μM KSR AS-ODN-1 elicited comparable reduction of endogenous KSR1 gene expression (over 80%) to that observed in PANC-1 cells, as determined by Western blot analysis (FIGURE 13B). Nonetheless, inhibition of proliferation was observed only in PANC-1 cells (FIGURE 13A). Equal loading of the gels was confirmed using total P44/42 MAPK. Note that purified Flag-KSR, which served as a positive control for the Western blot, migrates slightly slower than endogenous KSR due to the Flag-tag (FIGURE 13B). These results provide evidence that *gf* Ras signaling is specifically coupled to KSR1.

EXAMPLE 5

[0210] The full length mRNA sequence of human KSR1 has been determined. The sequence is depicted in FIGURE 14 (SEQ ID NO: 24). Antisense oligonucleotides, including those described above, were designed against the CA1 domain of human KSR nucleic acid. The human KSR antisense oligonucleotides are depicted on the annotated human KSR sequence in FIGURE 15. Oligonucleotide AS-ODN1(187-204) (5'CTTTGCCTCTAGGGTCCG 3') (SEQ ID NO: 28) against nucleotides 187 to 204 of the human sequence corresponds in sequence to AS-ODN1(214-231) (SEQ ID NO:8) described above. Thus, AS-ODN1 is complementary to the nucleotides at positions 187-204 and 214-231 of the human and mouse cDNA, respectively. Oligonucleotide AS-ODN3(124-141) (5'CAGCCCGCGCAGACTGCC 3') (SEQ ID NO: 29) against nucleotides 124 to 141 of the human sequence corresponds in sequence to AS-ODN1(151-168) (SEQ ID NO: 6) described above. Thus, AS-ODN3 is complementary to the nucleotides at positions 124-141 and 151-168 of the human and mouse cDNA sequences, respectively. Oligonucleotide AS-ODN2(154-171) is designed against nucleotides 154 to 171 of the human sequence. AS-ODN2 is complementary to nucleotides at positions 154-171 and 181-198 of the human and mouse cDNA, respectively. The human sequence differs by a single base pair in the most 5' bp of the antisense sequence from the mouse sequence in the corresponding position, with the human AS-ODN2(154-171) sequence being 5'GAGGTCGTTAGACACTG**C** 3' (SEQ ID NO: 30) and the mouse sequence being 5'GAGGTCGTTAGACACTG**A** 3' (SEQ ID NO: 7) (the nucleotide difference is set out in bold). FIGURE 16 depicts the annotated mouse KSR cDNA sequence with antisense oligonucleotides indicated. We have compared the original AS-ODN2(181-198) to the revised human AS-ODN2(154-171) and found they inhibited proliferation of PANC-1 cells, which are oncogenic K-*ras*-dependent human pancreatic cells, nearly identically (FIGURE 18).

EXAMPLE 6

[0211] We have designed additional potential AS-ODNs against other nucleotides of human KSR1. These ODNs (AS-ODN4 to AS-ODN12) are marked and annotated on human KSR1 nucleotide sequences in FIGURE 17. TABLE 2 is a list of these newly designed ODNs with corresponding human nucleotide target sequence.

TABLE 2

AS-ODN ID #	Target Sequence (nt) (5'-3')	Sequence (5' to 3')
AS-ODN4	ATGGGAGAGAAGGAGGGC (1–18)	GCCCTCCTTCTCTCCCAT (SEQIDNO: 31)
AS-ODN5	CTGGTCCGTTACATTTGT (205-222)	ACAAATGTAACGGACCAG (SEQ ID NO: 32)
AS-ODN6	GTGGCTCCCGGTGAGAGG (247–264)	CCTCTCACCGGGAGCCAC (SEQ ID NO: 33)
AS-ODN7	GACTGGCTGTACACTTTC (298–315)	GAAAGTGTACAGCCAGTC (SEQ ID NO: 34)
AS-ODN8	GAGGCCGGAGGTGGTGCA (321-338)	TGCACCACCTCCGGCCTC (SEQ ID NO: 35)
AS-ODN9	AGATCCCCCGAGACCTCA (351-368)	TGAGGTCTCGGGGGATCT (SEQ ID NO: 36)
AS-ODN10	ATGAATGAGGCCAAGGTG (379-396)	CACCTTGGCCTCATTCAT (SEQ ID NO: 37)
AS-ODN11	AGTTGGAGTTCATTGGAT (511-528)	ATCCAATGAACTCCAACT (SEQ ID NO: 38)
AS-ODN12	GCGGCGGGAAAGTGGCTC (531-548)	GAGCCACTTTCCCGCCGC (SEQ ID NO: 39)

EXAMPLE 7

KSR ANTISENSE OLIGONUCLEOTIDES AS RADIOSENSITIZERS

BACKGROUND

[0212] Although ionizing radiation (IR) remains a primary treatment for human cancers, the failure to respond to radiation therapy limits the efficacy of this modality. Accumulating evidence supports the contention that a signal transduction pathway, analogous to that for cell growth and differentiation leads to resistance to IR. More specifically, constitutive activation of the EGFR/Ras pathway via *gf* Ras or hyperactivation of EGFRs lead to radioresistance in human tumor cells (1-27). The precise mechanisms underlying this action of EGFR and Ras are not well understood. Hence, identification of downstream elements of EGFR and Ras effector signaling in response to IR is critical to the development of mechanism-based therapeutic strategies for IR treatment. Of the EGFR and Ras effector signaling pathways, the Ras-Raf-MAPK (10, 28-32) and Ras-PI3-Kinase (3,33,34) pathways have been implicated to mediate EGFR/Ras regulation of radiosensitivity to IR. The relative contribution of these two signaling pathways may be tumor-type specific.

[0213] Potential mechanisms underlying EGFR/Ras-mediated radioresistance include: increased capacity for DNA damage repair, accelerated repopulation of surviving tumor cells, resistance to IR-induced apoptosis and lack of IR-induced cell cycle check point delays. IR-induced EGFR/Ras activation results in a pronounced dose-dependent proliferative response via the MAPK signaling cascade, contributing at least in part, to the mechanism of accelerated proliferation, a cause contributing to the failure of radiotherapy (49,50). As an obligate mediator of the *gf* EGFR/Ras-Raf-1-MAPK signaling of tumor cell proliferation (51), inactivation of KSR may increase the efficacy of IR therapy by abrogating the accelerated repopulation of surviving tumor cells.

[0214] In addition to potentially mediating radiation resistance through EGFR and Ras, KSR may also transmit signals for the lipid second messenger ceramide. Emerging data suggest that IR acts directly on the plasma membrane of several cell types, activating acid sphingomyelinase, which generates ceramide by enzymatic hydrolysis of sphingomyelin. Ceramide then acts as a second messenger in initiating or inhibiting an apoptotic response that is cell-type and cell-context specific (13, 15, 35-38). In addition to the tyrosine kinase based mechanism that the EGFR utilizes for KSR activation, ceramide may also directly activate KSR. In this regard, KSR was originally identified by Kolesnick and co-workers as ceramide activate protein kinase (CAPK) (39). While ceramide can signal apoptosis in some cell types through the c-Jun kinase and transcriptional regulation of gene products, such as Fas ligand or TNFα that mediate the death response (13, 14, 45), in other cells that contain the pro-apoptotic Bcl-2 family member BAD, ceramide induces apoptosis directly through a mechanism involving sequential Ras-dependent activation of KSR, c-Raf-1, and MEK1 (44, 46-48). In this case, the availability of a single target, such as BAD (44), converts the MAPK cascade, which is usually proliferative and/or anti-apoptotic, into a pro-apoptotic signaling pathway. Therefore, KSR may modulate radiosensitivity by regulation of ceramide-dependent and independent apoptotic responses in a cell-type specific manner. In support of the notion that KSR may prevent some forms of apoptosis from ensuing, Polk and co-workers showed that inactivation of KSR in YAMC colon cells antagonizes the anti-apoptotic signals emanating from TNF receptor, converting TNF into an inducer of apoptosis in these cells (41,42).

[0215] In the present example, we confine our studies to inactivation of the EGFR/Ras pathway as a proof-in-principle that this strategy can lead to radiosensitization. To explore this venue, we synthesized KSR specific AS-ODNs to pharmacologically inhibit KSR function (as described herein above) and employed A431 cells, which are driven through wild type Ras by an 100-fold overexpression of activated EGFR. When tested *in* *vitro*, AS-ODNs were specifically taken up into the nucleus, resulting in decreased endogenous KSR gene expression and inhibition of A431 proliferation, invasion, transformation and tumorigenesis (see Example 2). Moreover, here we demonstrate that inactivation of KSR via genetic (expression of KSR-AS) or pharmacological (AS-ODNs) approaches yields sensitization of A431 cells to IR-induced apoptosis *in vitro*.

RESULTS

[0216] AS-KSR and DN Ki-KSR sensitize A431 cells to IR-induced apoptosis: To test the hypothesis that inactivation of KSR might enhance the sensitivity to IR-induced cell killing, A431 cells stably-transfected with different KSR constructs were analyzed for their sensitivity to IR-induced apoptosis. A431 cells, due to the overexpression of activated EGFR, are known to be radioresistant (43). Serum-starved A431 cells were irradiated with a single dose of 20Gy and apoptosis was determined 24 hr post-IR by flow cytometry using Annexin V-FITC (Sigma). FIGURE 19 shows that A431 cells overexpressing wild-type Flag-KSR (KSR-S) displayed minimal IR-induced apoptosis. Expression of Flag-AS-KSR (KSR-AS) or dominant-negative Flag-Ki-KSR (Ki-KSR) radiosensitized A431 cells, resulting in a substantial increase in IR-induced apoptosis (n=3). Nearly 40% of A431- KSR-AS and Ki-KSR cells underwent apoptosis 24 hr after IR. These results, although preliminary, strongly suggest that KSR may play a key role in cellular sensitivity to IR. Comparable results were obtained when apoptosis was scored with Bisbenzimide staining.

KSR AS-ODNs sensitize A431 cells to IR-induced apoptosis:

[0217] As described above, abrogation of KSR function by a genetic approach (AS-KSR and DN-Ki-KSR) sensitized A431 cells to IR-induced apoptosis. To test the effectiveness of pharmacologic inhibition of KSR1 gene expression by AS-ODN on radiosensitivity, A431 cells were treated with 200 nM of AS-214231 for 36 hr prior to IR. Apoptosis was quantitated by flow cytometry using Annexin V-FITC. As shown in FIGURE 20, AS-214231 sensitized A431 cells, leading to a 2-fold increase in IR-induced apoptosis after 72 hours. The magnitude of this effect is comparable to that observed with overexpression of AS-KSR. In contrast, control ODN had no significant effect on radiation sensitivity to IR-induced apoptosis. It should be noted that at the concentration of Oligofectamine used, no significant elevation of basal level of apoptosis was detected (not shown). These results indicate that the radiosensitizing effect of AS-214231 is KSR sequence specific and that it might be feasible to use KSR AS-ODNs as radiosensitizers.

[0218] Inactivation of KSR1 by KSR-AS inhibits A431 cells to IR-induced clonogenic survival: To assess whether KSR1 is involved in the lethal effects of ionizing radiation on A431 cells, the clonogenic survival of KSR-S- and KSR-AS-transfected A431 cells was determined after increasing doses of radiation exposure. A431-pTRE, KSR-S and KSR-AS cells were plated at seeding density of 3.4 x 10^4, 1.4 x 10^4, or 7.0 x 10^3 cells/cm^2, respectively in 60 mm dishes for 4 days to reach 85% confluency. Prior to radiation, the culture medium was replaced with serum-free growth medium containing 0.2% human albumin for 20-24 hr. Cultures were then subjected to 1-15Gy of radiation at a dose rate of 2.24Gy/minute, using a 60^{Co} source. The culture medium was then replaced again with fresh growth medium containing 10% Tet-approved FBS for additional 24 h. Thereafter, cells were harvested by trypsinization and a clonogenic survival assay was performed using a standard procedure. The cells were seeded at different dilutions to generate 25-80 colonies per 60 mm culture dish for each radiation dose, incubated at 37°C in 5% CO_2 for 12 days and colonies containing over 50 cells were stained with crystal violet to determine the surviving fractions. FIGURE 21 demonstrates that overexpression of KSR-S mildly enhanced radioresistance as compared to A431-pTRE controls, associated with an increase in the shoulder width (D_q) of the dose-survival curve, without a significant change in the slope (D_0). In contrast, inactivation of KSR1 by KSR-AS significantly radiosensitized A431 cells, manifested by complete elimination of the dose-survival curve shoulder and a decrease of the D_0. The dose modification factor (MDF) at 10% survival level (D_{10}) upon KSR inactivation was 2.44. These results indicate that KSR1 is obligate for radiation-induced cell kill and that KSR1 represent a new molecular target for radiosensitization.

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EXAMPLE 8

Regulation of angiogenesis by KSR1

[0219] Background: Angiogenesis, the formation of new vessels from pre-existing vasculature, plays a crucial role during normal development, tissue regeneration in wound healing and post-ischemic tissue repair. In addition, angiogenesis is central to tumor growth, progression and dissemination[a,b,c,d]. New vessel growth and maturation are highly complex and coordinated processes, requiring the sequential activation of a series of receptors by numerous ligands[e,f]. Vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis. VEGF signaling, mediated by two receptor tyrosine kinases VEGFR-1 and VEGFR-2, represents a critical rate-limiting step not only in physiologic angiogenesis such as embryogenesis, skeletal growth and reproductive functions, but also in pathological angiogenesis, such as that associated with tumor growth[g].

[0220]Through alternative mRNA splicing, human VEGF gene gives rise to four major VEGF isoform ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{201}$) having respectively 121, 163, 189 and 201 amino acids after signal sequence cleavage [h,i]. While $VEGF_{121}$ is a free diffusible protein, $VEGF_{189}$, and $VEGF_{206}$ have high affinity for heparin and are mostly bound to heparin-containing proteoglycans in the extracellular matrix (ECM)[j,k]. VEGF165, the predominant isoform, is secreted yet with a significant portion remain bound to cell surface or ECM[k]. The biological functions of VEGF are mediated by two receptor tyrosine kinases VEGFR-1 (also known as Flt-1) and VEGFR-2 (also known as Flk-1 or KDR). VEGFR-2 is the chief mediator of VEGF signaling of endothelial cell mitogenesis and survival, angiogenesis and microvascular permeability. In contrast, VEGFR-1 does not transmit mitogenic signal effectively. It inhibits VEGFR-2 activity by sequestering VEGF and prevents it from binding VEGFR-2. In addition, VEGFR-1 modulates the release of tissue-specific growth factors from vascular bed[f].

[0221]The gene expression of VEGF is tightly regulated. VEGF mRNA expression is up regulated through hypoxia-induced factor (HIF) in response to hypoxic or oxygen tension[l]. In addition, hyperactivation of receptor tyrosine kinases such as EGFR or oncogenic activation of Ras leads to transcriptional upregulation of VEGF expression[g,m]. Further, DePinho and colleagues recently demonstrate that oncogenic H-Ras signaling is obligate for melanoma tumor maintenance via regulation of VEGF production and endothelial cell survival[n]. Moreover, importance of Ras-Raf-1 in angiogenesis is strengthened by the observations of Cheresh and co-workers that Raf-1 confers vascular survival in response to intrinsic and extrinsic apoptotic stimuli[o].

[0222]The importance of VEGF in angiogenesis has established it as a therapeutic target for cancer treatment and led to the clinical development of a variety of VEGF inhibitors.

In 1993, it was shown that a monoclonal antibody that targeted VEGF results in a dramatic suppression of tumor growth *in vivo*[p], which led to the development of bevacizumab (Avastin; Genentech), a humanized variant of this anti-VEGF antibody, as an anticancer agent. The recent approval of bevacizumab by the US FDA as a first-line therapy for metastatic colorectal cancer validates the ideas that VEGF is a key mediator of tumor angiogenesis and that blocking angiogenesis is an effective strategy to treat human cancer[q].

Results: KSR1 is required for VEGF production in A431 cells

[0223]Since KSR1 is an essential mediator of gain-of-function Ras signaling of tumorigenesis[r,s], we evaluated the involvement of KSR1 in EGF-stimulated VEGF production in A431-pTRE-Tet-Off-KSR cell lines that we have previously characterized[s]. For these studies, A431-pTRE-KSR cells were seeded at 6 x 10^5 cells (for pTRE and KSR-S cells) or 3 x 10^5 cells (for KSR-AS and DN-KSR cells), respectively in 6-well culture plates and let grow for 48 hr to 80% confluent. Cells were then washed twice with PBS and incubated in 1ml serum-free medium in the presence or absence of EGF (1-100 ng/ml) for additional 24 or 48 hr. At the indicated time points, conditioned medium containing secreted forms of VEGF was collected, cell debris cleared by centrifugation and stored at –80°C until further characterization. ELISA immunoassay (R&D Systems) was performed to measure soluble forms of VEGF in conditioned medium utilizing an antibody recognizing all isoforms of VEGF according to manufacturer's instructions. FIGURE 22 shows that approximately 400 pg/ml/10^5cells VEGF were secreted by A431-pTRE cells over 24h, consistent with the reported value in the literature[t]. Overexpression of Flag-KSR1 in A431-KSR-S cells lead to a 3-fold increase of basal VEGF production compared to that of A431-pTRE cells. In contrast, inactivation of KSR1 by either KSR-AS or a dominant negative KSR1 mutant (DN-KSR1) completely abolished baseline VEGF secretion at all time points examined. Furthermore, while VEGF in non-transfected A431 cells or in A431-pTRE and KSR-S cells could be further increased by stimulation with 100 ng/ml EGF treatment, A431-KSR1-AS and A431-DN-KSR1 cells failed to respond to EGF stimulation. Similar results were observed when stimulated with lower doses of EGF. These results suggest that KSR1 is obligate for baseline and EGF-induced VEGF ligands production *in vitro* and KSR1 may represent a novel target for anti-angiogenic therapy to treat conditions associated with pathological angiogenesis such as cancer. Future studies will evaluate the effectiveness of KSR1 AS-ODN in inhibiting VEGF expression in A431 cells *in vitro* and in A431 tumor xenografts *in vivo*.

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[0224] This invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all aspects illustrate and not restrictive, the

2004297594 16 Sep 2009

scope of the invention being indicated by the appended Claims, and all changes which come within the meaning and range of equivalency are intended to be embraced therein.

[0225] All publications mentioned in this specification are herein incorporated by reference. Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia or elsewhere before the priority date of each claim of this application.

[0226] Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of conferring radiosensitivity to ionizing radiation in tumor cells in a mammal comprising administering to a mammal a therapeutically effective amount of a compound or agent which inhibits the expression of mammalian KSR protein.

2. A method of conferring radiosensitivity to ionizing radiation in tumor cells in a mammal comprising administering to a mammal a therapeutically effective amount of an antisense oligonucleotide, wherein the antisense oligonucleotide is specifically hybridizable to a portion of the coding region of mRNA encoding human KSR (SEQ ID NO:24).

3. A method of inhibiting angiogenesis of tumor cells in a mammal comprising administering to a mammal a therapeutically effective amount of a compound or agent which inhibits the expression of mammalian KSR protein.

4. The method of any one of claims 1 to 3, wherein the tumor cells are cancer cells selected from the group consisting of pancreatic cancer, lung cancer, skin cancer, urinary tract cancer, bladder cancer, liver cancer, thyroid cancer, colon cancer, intestinal cancer, leukemia, lymphoma, neuroblastoma, head and neck cancer, breast cancer, ovarian cancer, stomach cancer, esophageal cancer and prostate cancer.

5. A method of inhibiting angiogenesis in a mammal comprising administering to a mammal a therapeutically effective amount of an antisense oligonucleotide, wherein the antisense oligonucleotide is specifically hybridizable to a portion of the coding region of mRNA encoding human KSR (SEQ ID NO:24).

6. A method for inhibiting or reducing VEGF expression or activity in a mammal comprising administering to a mammal a therapeutically effective amount of a compound or agent which inhibits the expression of mammalian KSR protein.

7. A method for inhibiting or reducing VEGF expression or activity in a mammal comprising administering to a mammal a therapeutically effective amount of an antisense oligonucleotide, wherein the antisense oligonucleotide is specifically hybridizable to a portion of the coding region of mRNA encoding human KSR (SEQ ID NO:24).

8. The method of any one of claims 5 to 7, wherein the inhibition of angiogenesis is determined by a decrease in the expression of VEGF.

9. The method of any one of claims 2, 5, 7 and 8, wherein the antisense oligonucleotide comprises a sequence specifically hybridizable to a nucleic acid encoding the CA1 region, amino acids 33 to 72 of human KSR (SEQ ID NO:26), or a portion thereof.

10. The method of any one of claims 2, 5, 7 and 8, wherein the antisense oligonucleotide comprises a sequence specifically hybridizable to a nucleotides 97 to 216 of human KSR (SEQ ID NO:25), or a portion thereof.

11. The method of any one of claims 2, 5, 7 and 8, wherein the antisense oligonucleotide comprises a sequence specifically hybridizable to a nucleotides 124 to 141 (SEQ ID NO:3), 154 to 171 (SEQ ID NO: 27), or 187 to 204 (SEQ ID NO:5) of the sequence of human KSR.

12. The method of any one of claims 2, 5, 7 and 8, wherein the antisense oligonucleotide comprises a sequence specifically hybridizable to a portion of the coding region of human KSR (SEQ ID NO:24) selected from:

a. Nucleotides 1 to 18 (ATGGGAGAGAAGGAGGGC);
b. Nucleotides 205 to 222 (CTGGTCCGTTACATTTGT);
c. Nucleotides 247 to 264 (GTGGCTCCCGGTGAGAGG);
d. Nucleotides 298 to 315 (GACTGGCTGTACACTTTC);
e. Nucleotides 321 to 338 (GAGGCCGGAGGTGGTGCA);
f. Nucleotides 351 to 368 (AGATCCCCCGAGACCTCA);
g. Nucleotides 379 to 396 (ATGAATGAGGCCAAGGTG);
h. Nucleotides 511 to 528 (AGTTGGAGTTCATTGGAT); and
i. Nucleotides 531 to 548 (GCGGCGGGAAAGTGGCTC).

13. The method of any one of claims 2, 5, 7 and 8, wherein the antisense oligonucleotide comprises a sequence selected from the group of SEQ ID NOS:28-39.

14. The method of any one of claims 2, 5, 7 and 8, wherein the antisense oligonucleotide comprises a sequence of SEQ ID NO:28.

15. The method of any one of claims 2, 5, 7 and 8 to 14, wherein the antisense oligonucleotide is labeled with a detectable label.

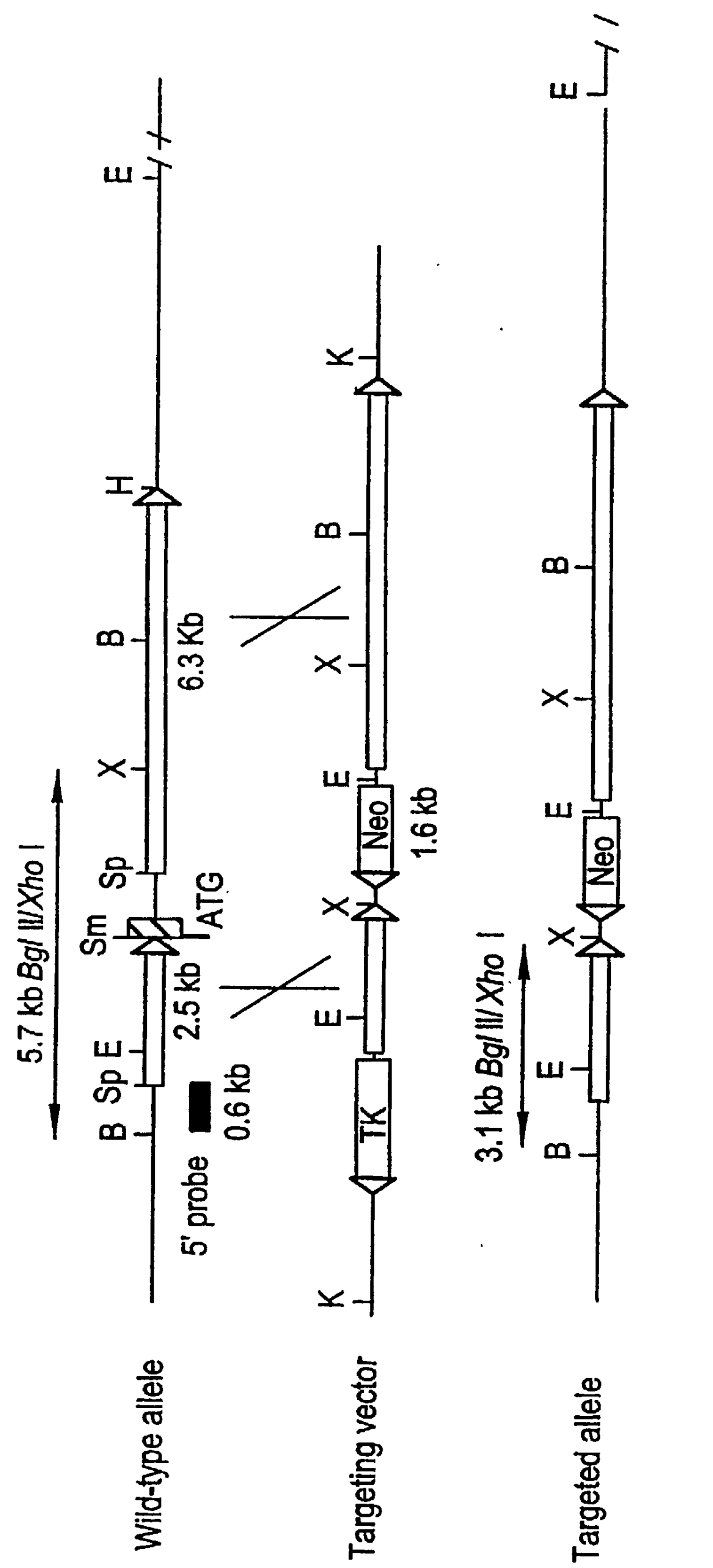
16. The method of claim 15, wherein the label is selected from enzymes, ligands, chemicals which fluoresce, or radioactive elements.

17. The method of any one of claims 2, 5, 7 and 8 to 14, wherein the antisense oligonucleotide is modified in its chemical backbone.

18. The method of claim 17, wherein the antisense oligonucleotide comprises at least one phosphorothioate linkage.

19. The method of claim 18, wherein the antisense oligonucleotide is a phosphorus deoxynucleotide.

FIG. 1A


Wild-type allele
Targeting vector
Targeted allele
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3.1 kb Bgl II/Xho I
6.3 Kb
2.5 kb
1.6 kb
0.6 kb
5' probe
ATG
Neo
TK
B
Sp
E
Sm
X
H
K

FIG. 1B

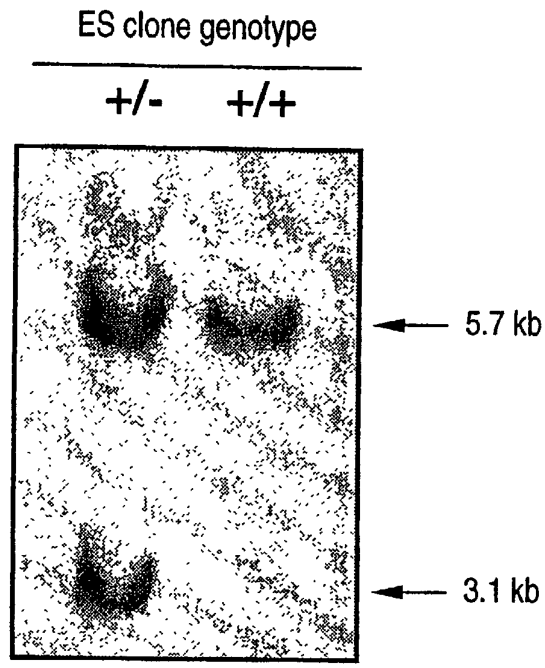


FIG. 1C

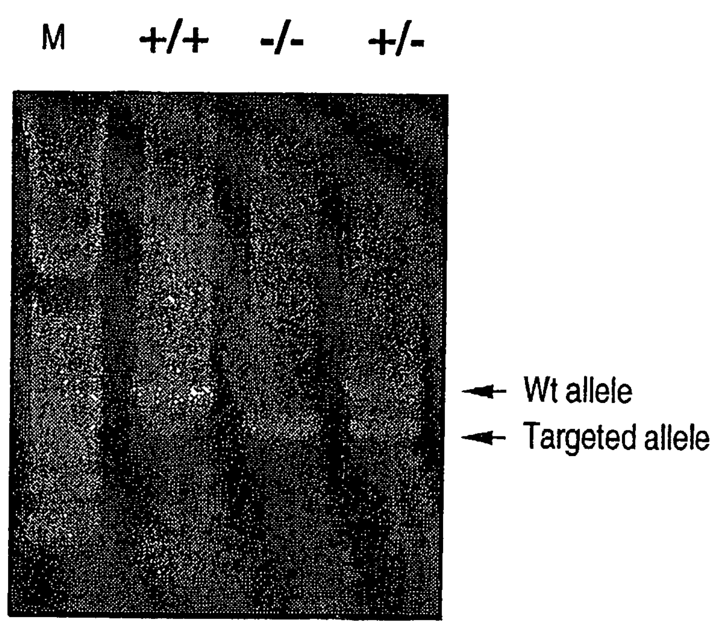


FIG. 1D

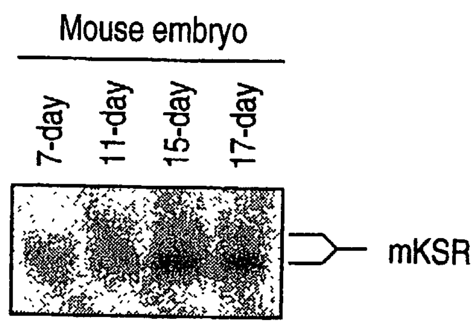


FIG. 1E

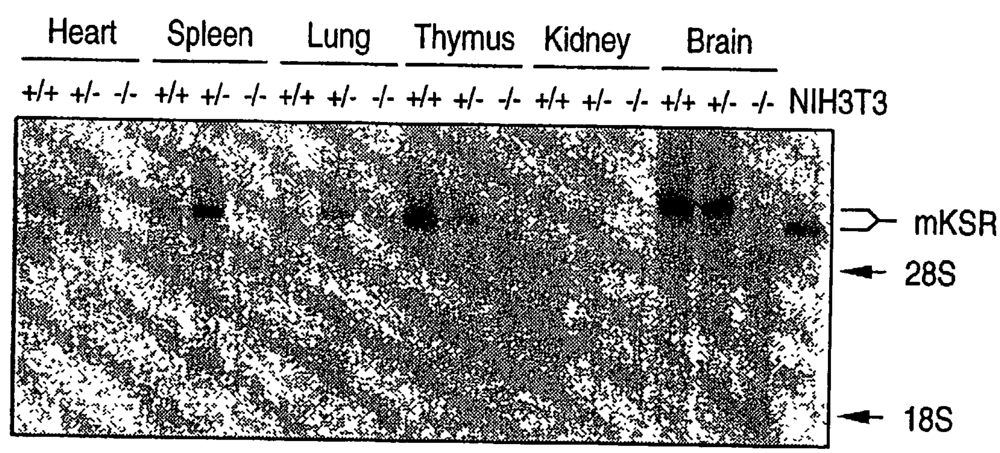


FIG. 1F

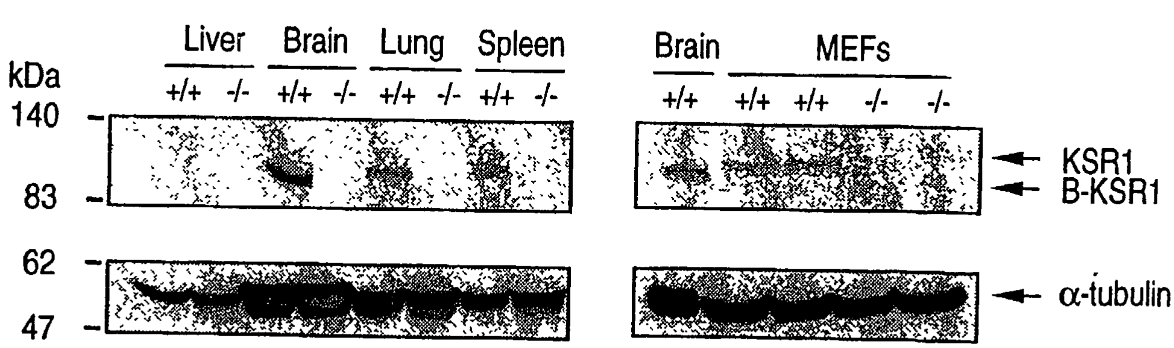


FIG. 2A

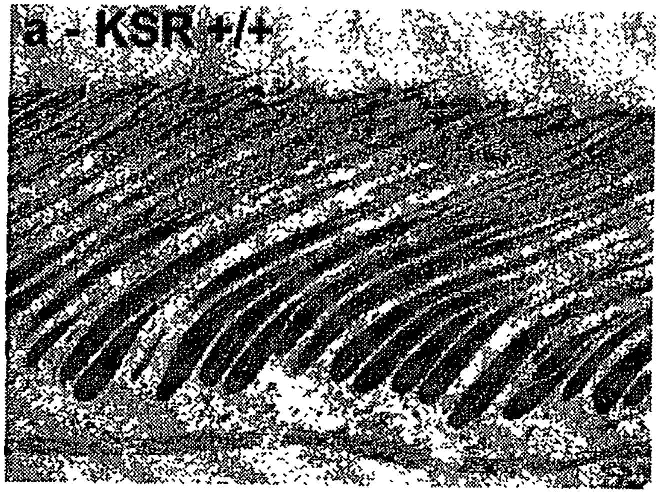


FIG. 2B

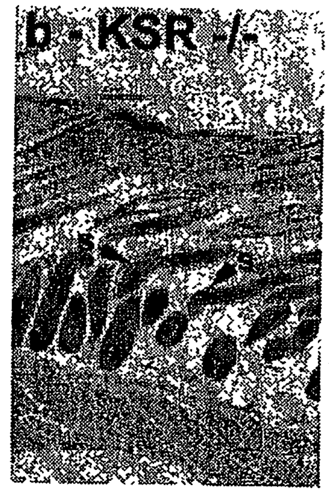
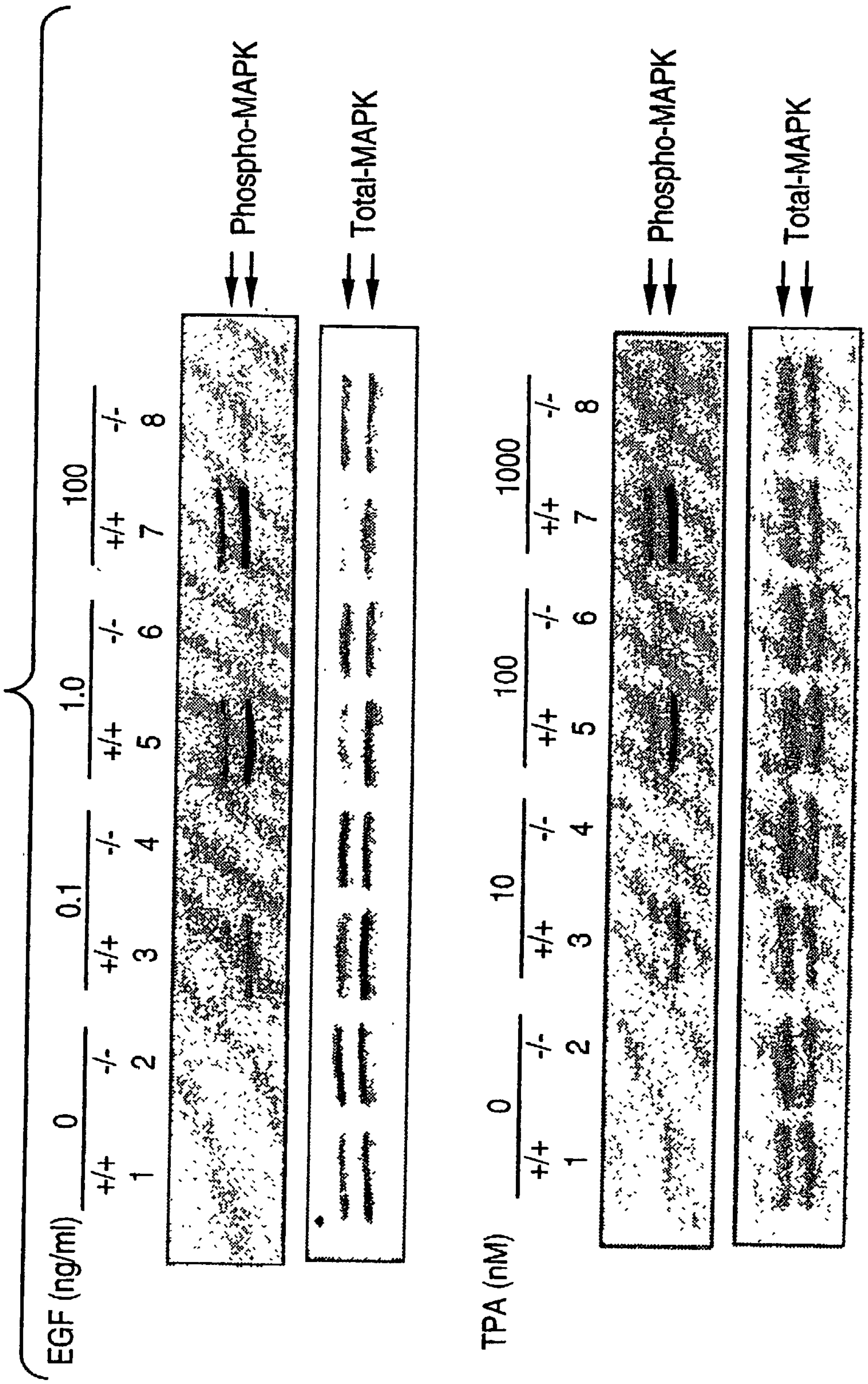


FIG. 2C

FIG. 2D

FIG. 3A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0.1
1.0
100
+/+
-/-
1
2
3
4
5
6
7
8
Phospho-MAPK
Total-MAPK
TPA (nM)
0
10
100
1000

FIG. 3B

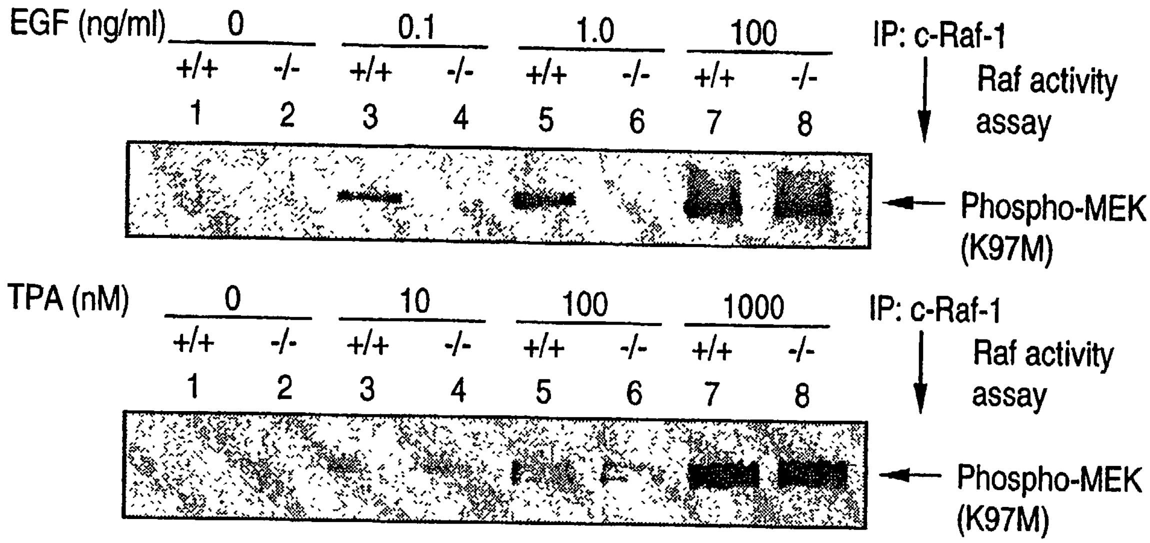

EGF (ng/ml)
0
0.1
1.0
100
+/+ -/- +/+ -/- +/+ -/- +/+ -/-
1 2 3 4 5 6 7 8
IP: c-Raf-1
Raf activity assay
Phospho-MEK (K97M)
TPA (nM)
0
10
100
1000
+/+ -/- +/+ -/- +/+ -/- +/+ -/-
1 2 3 4 5 6 7 8
IP: c-Raf-1
Raf activity assay
Phospho-MEK (K97M)

FIG. 3C

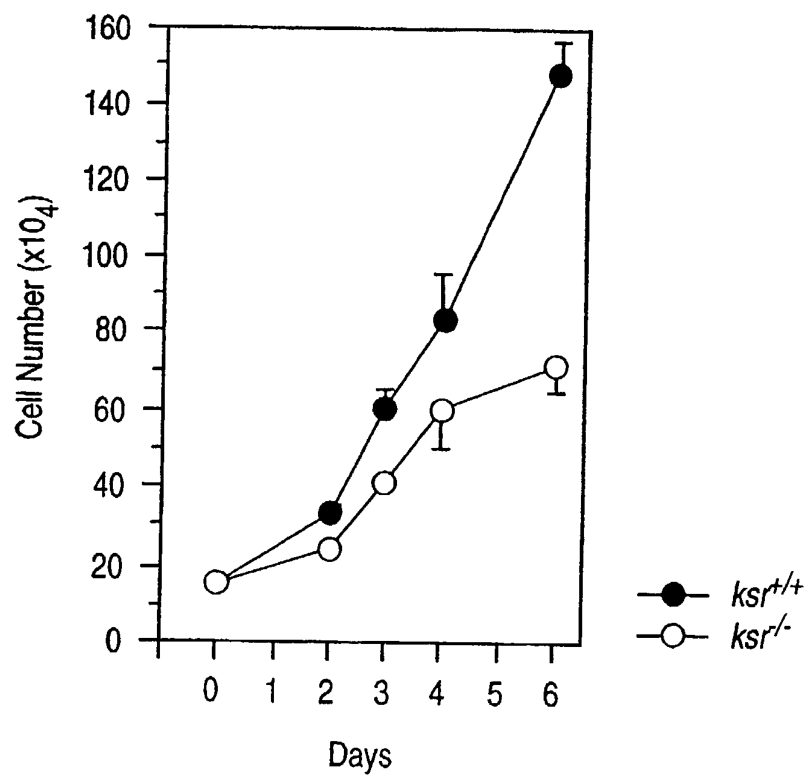

160
140
120
100
80
60
40
20
0
Cell Number (x10₄)
0 1 2 3 4 5 6
Days
ksr+/+
ksr-/-

FIG. 4A

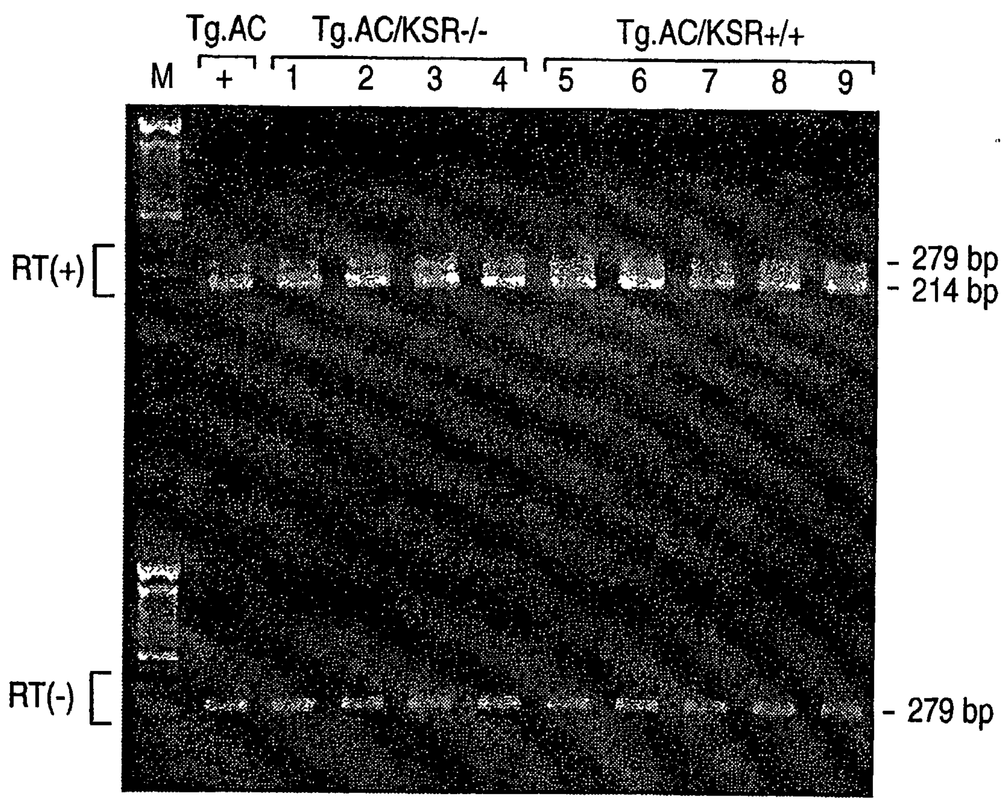


FIG. 4B

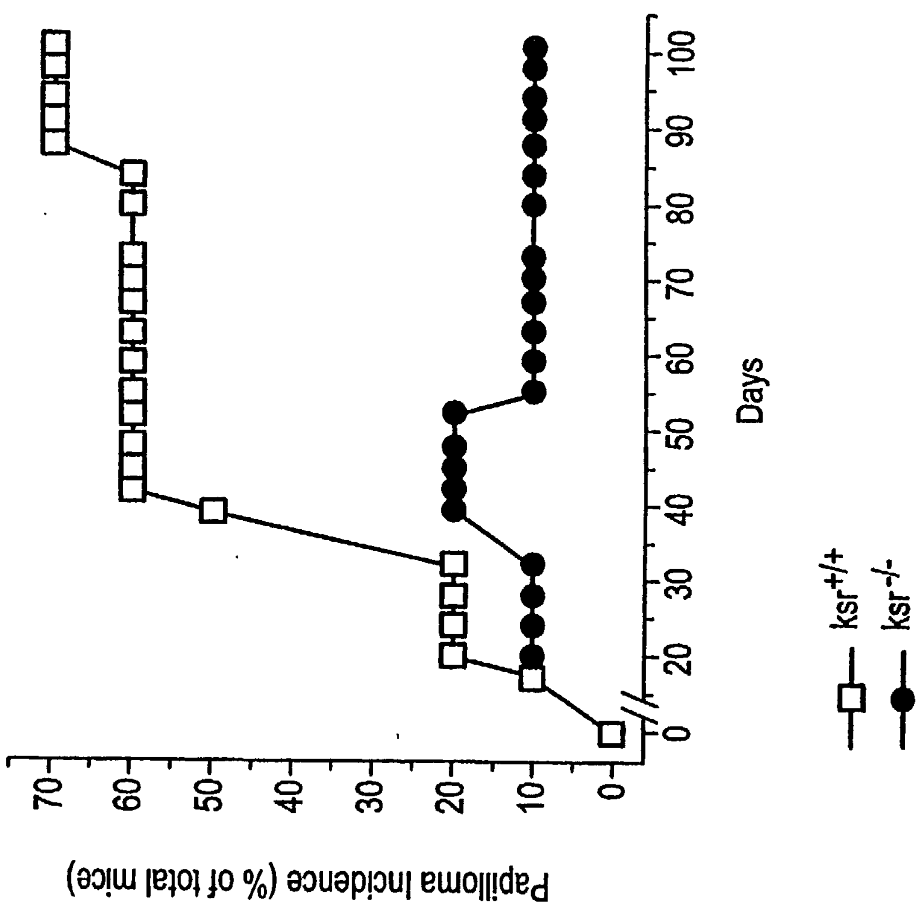

Papilloma Incidence (% of total mice)
70
60
50
40
30
20
10
0
0
20
30
40
50
60
70
80
90
100
Days
ksr+/+
ksr-/-

FIG. 5A

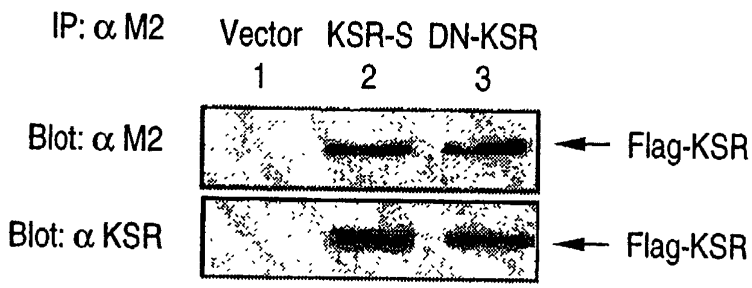


FIG. 5B

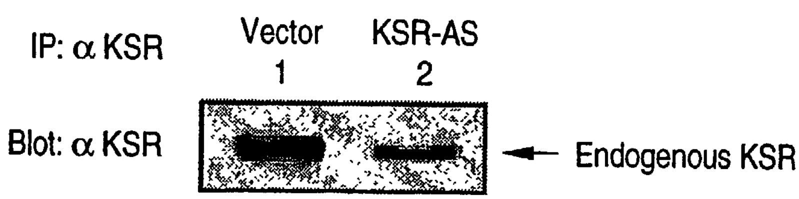


FIG. 5C

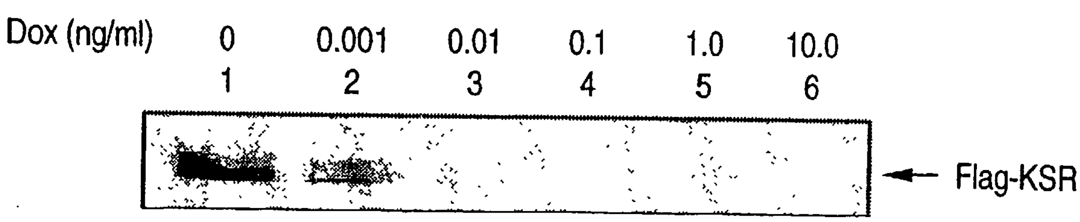


FIG. 5D

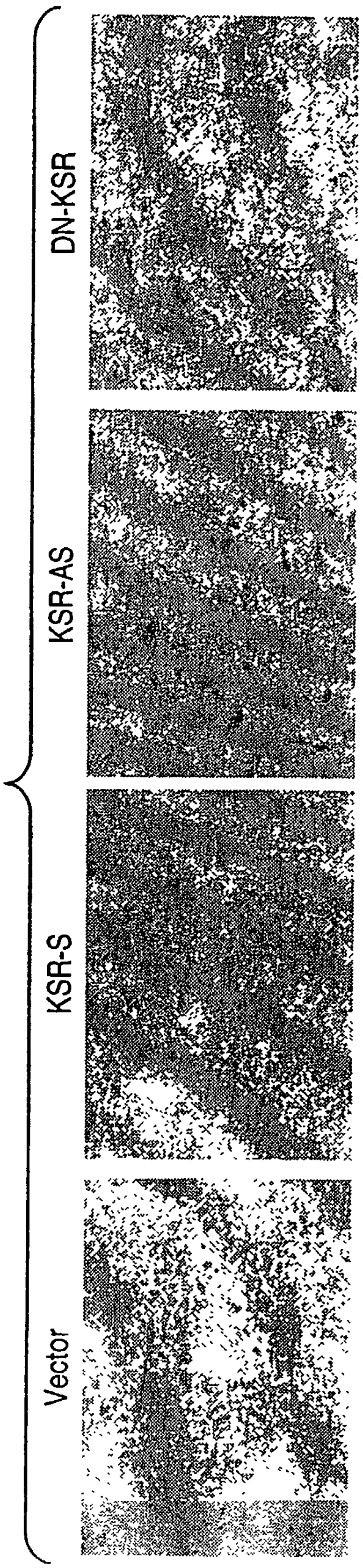


FIG. 5E

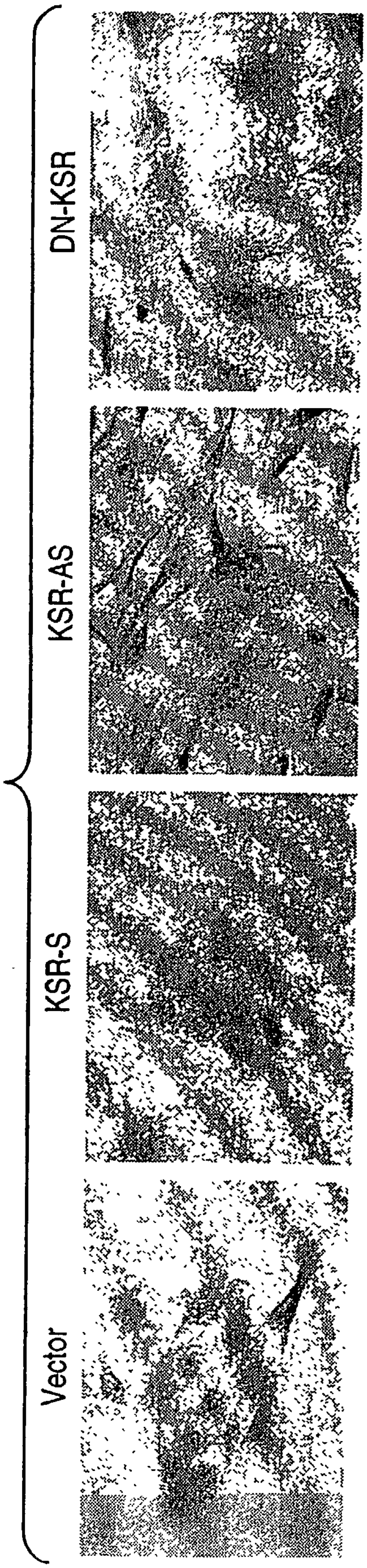


FIG. 6A

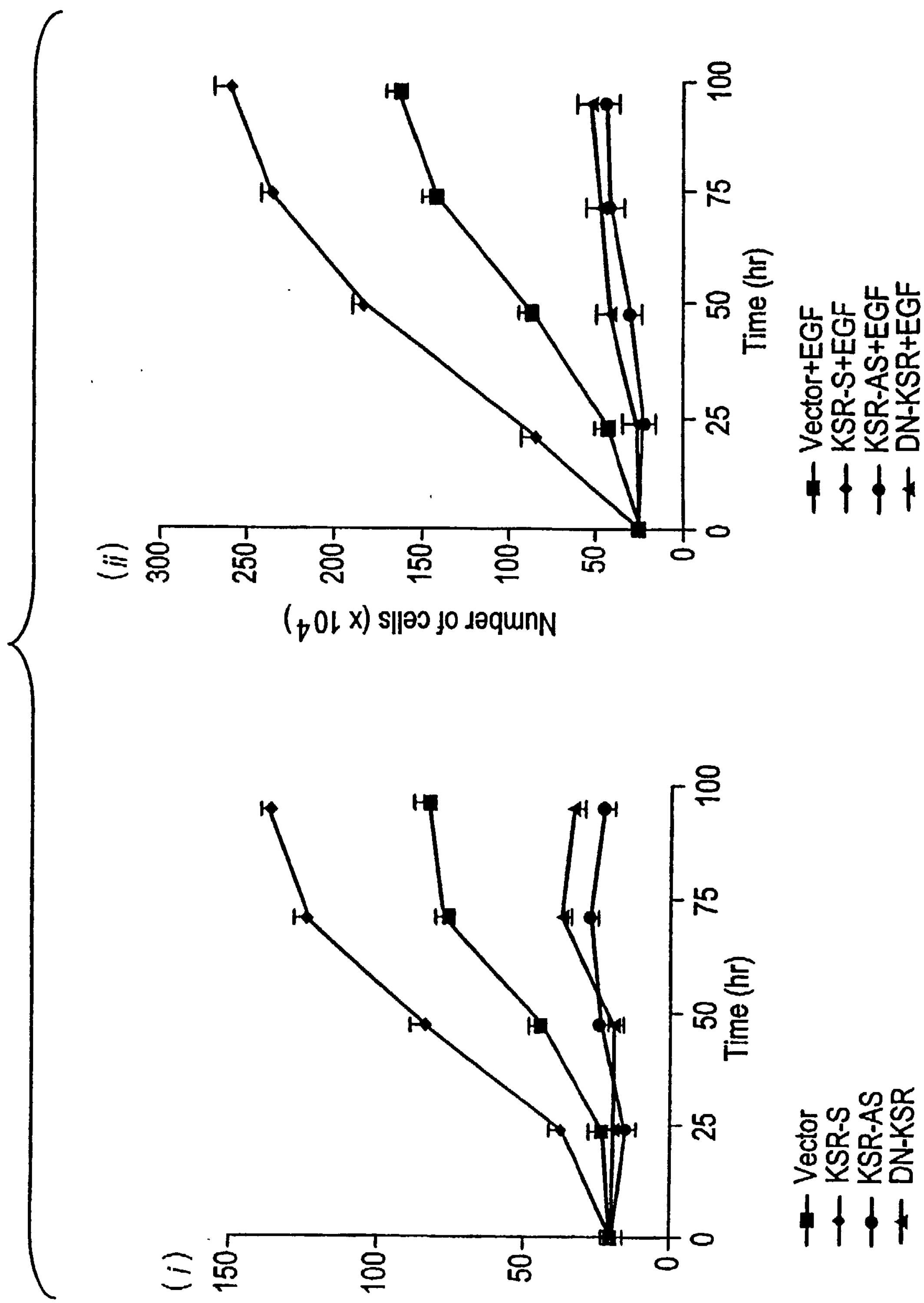

(i)
Number of cells (x 10⁴)
150
100
50
0
0
25
50
75
100
Time (hr)
Vector
KSR-S
KSR-AS
DN-KSR
(ii)
Number of cells (x 10⁴)
300
250
200
150
100
50
0
0
25
50
75
100
Time (hr)
Vector+EGF
KSR-S+EGF
KSR-AS+EGF
DN-KSR+EGF

FIG. 6B

	% G1	% S	% G2
Vector	40.1	45.1	14.8
KSR-S	25.2	60.8	14.0
KSR-AS	16.4	23.2	60.4
DN-KSR	24.2	24.8	51.0

FIG. 6C

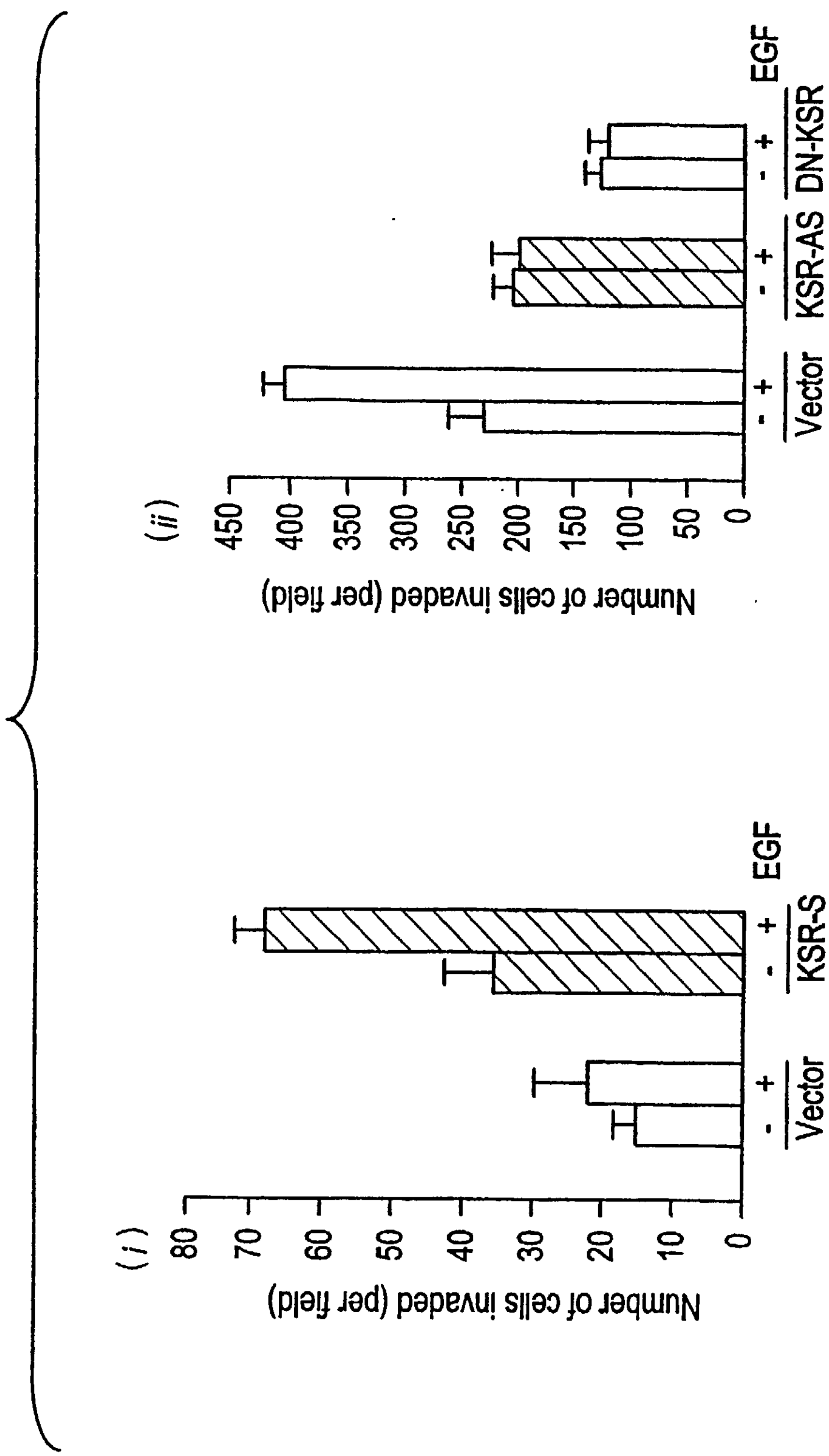

(i)
Number of cells invaded (per field)
80
70
60
50
40
30
20
10
0
- +
Vector
- +
KSR-S
EGF
(ii)
Number of cells invaded (per field)
450
400
350
300
250
200
150
100
50
0
- +
Vector
- +
KSR-AS
- +
DN-KSR
EGF

FIG. 6D

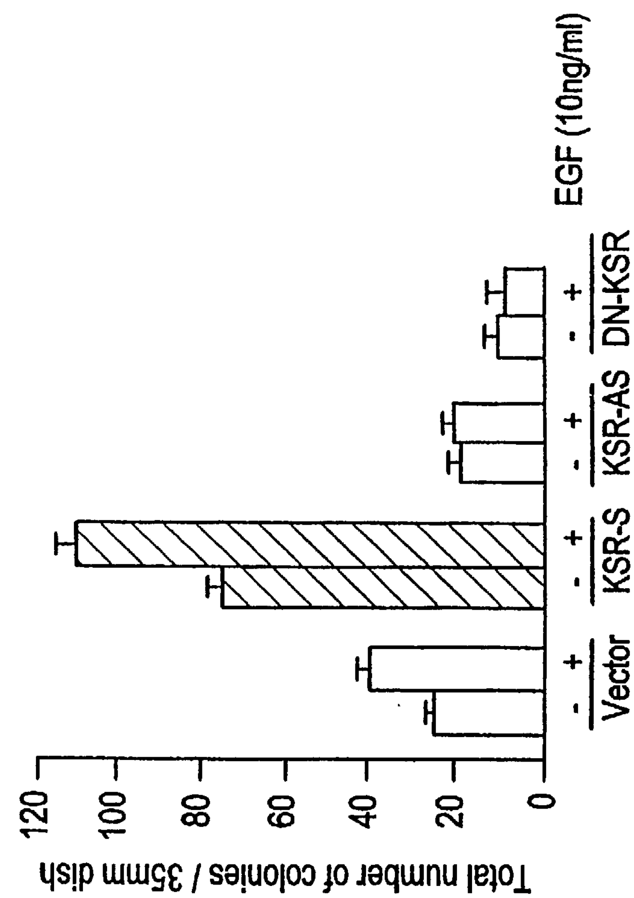

Total number of colonies / 35mm dish
0
20
40
60
80
100
120
-
+
Vector
-
+
KSR-S
-
+
KSR-AS
-
+
DN-KSR
EGF (10ng/ml)

FIG. 7A

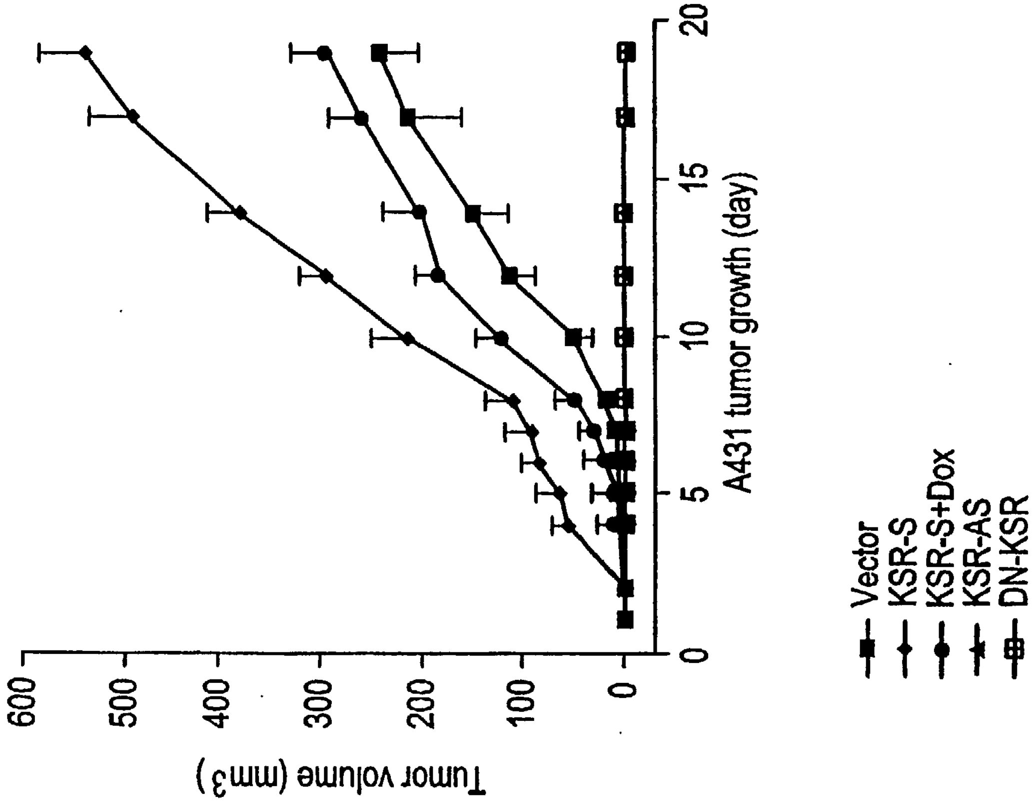
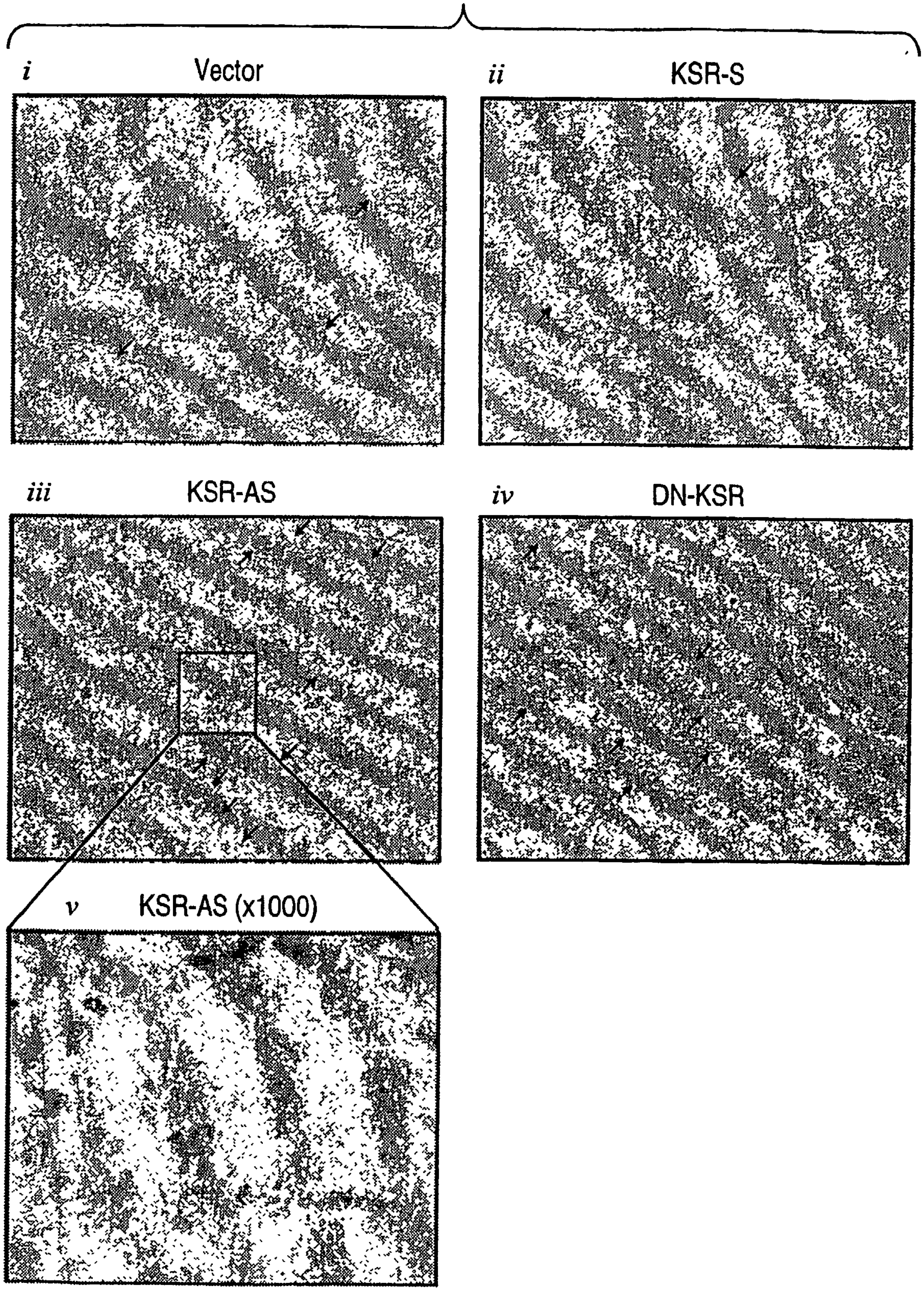

Tumor volume (mm³)
600
500
400
300
200
100
0
0
5
10
15
20
A431 tumor growth (day)
Vector
KSR-S
KSR-S+Dox
KSR-AS
DN-KSR

FIG. 7B



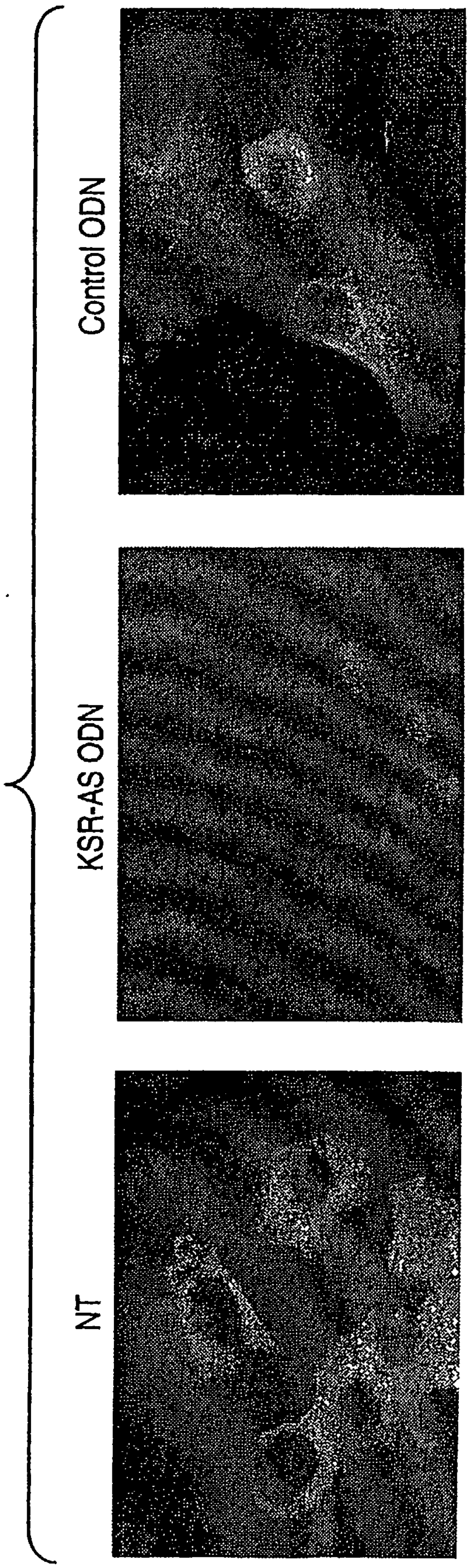


FIG. 8A

FIG. 8C

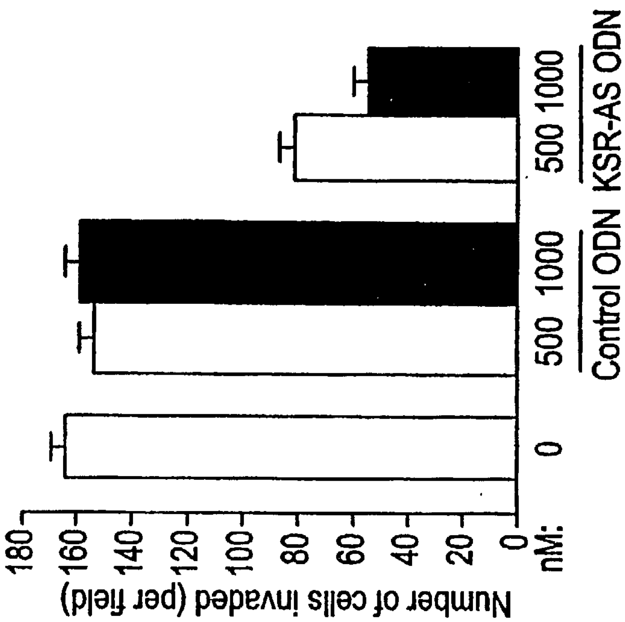

Number of cells invaded (per field)
180
160
140
120
100
80
60
40
20
0
nM:
0
500
1000
500
1000
Control ODN
KSR-AS ODN

FIG. 8B

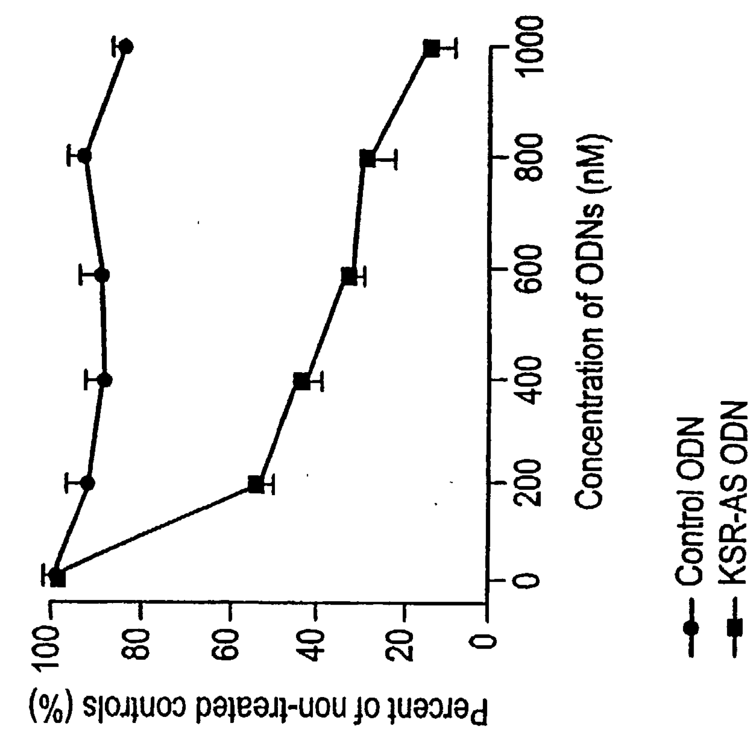

Percent of non-treated controls (%)
100
80
60
40
20
0
0
200
400
600
800
1000
Concentration of ODNs (nM)
Control ODN
KSR-AS ODN

FIG. 8D

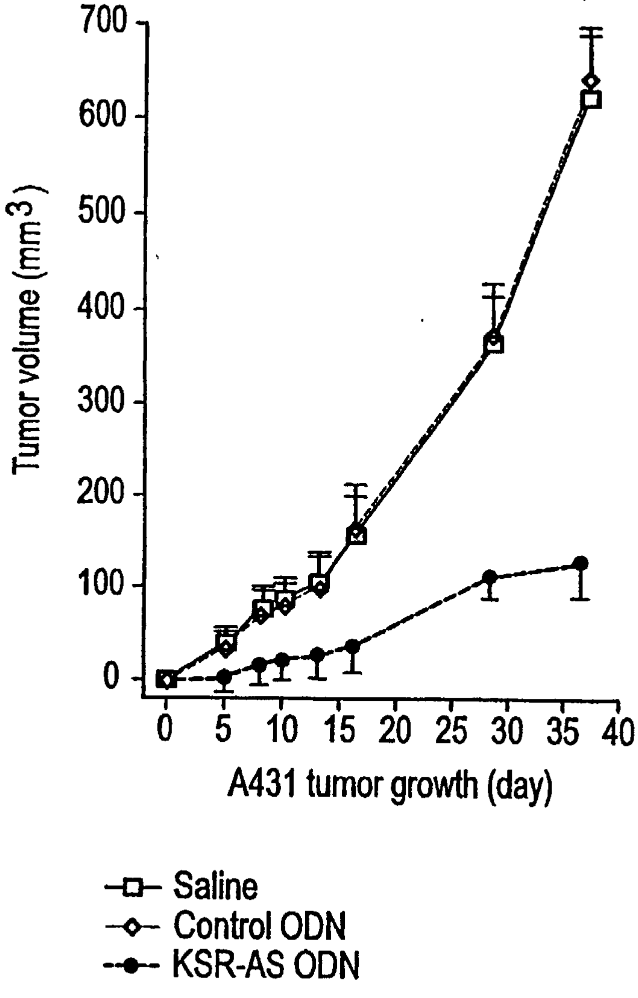

700
600
500
400
300
200
100
0
Tumor volume (mm3)
0 5 10 15 20 25 30 35 40
A431 tumor growth (day)
Saline
Control ODN
KSR-AS ODN

FIG. 9A

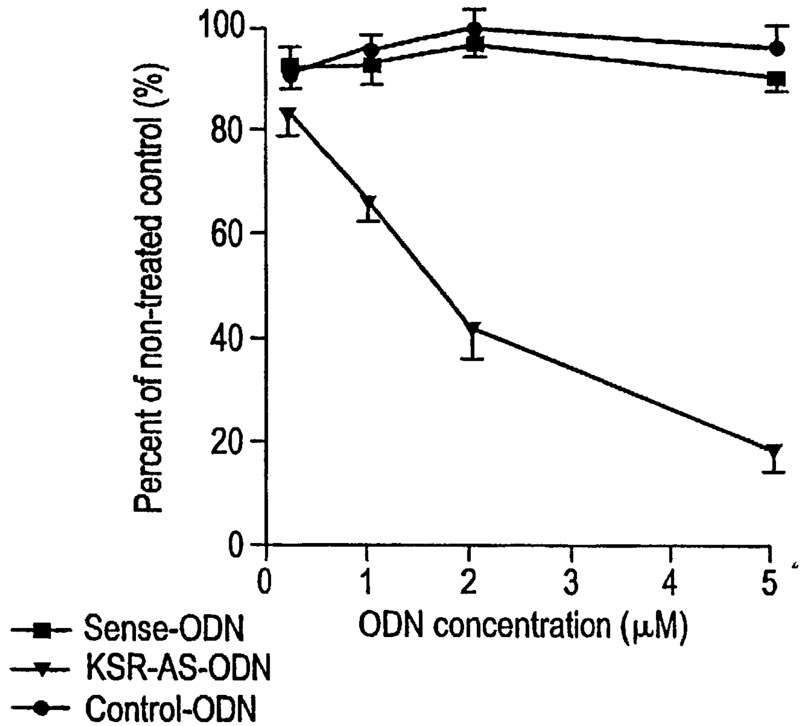

100
80
60
40
20
0
Percent of non-treated control (%)
0
1
2
3
4
5
ODN concentration (μM)
Sense-ODN
KSR-AS-ODN
Control-ODN

FIG. 9B

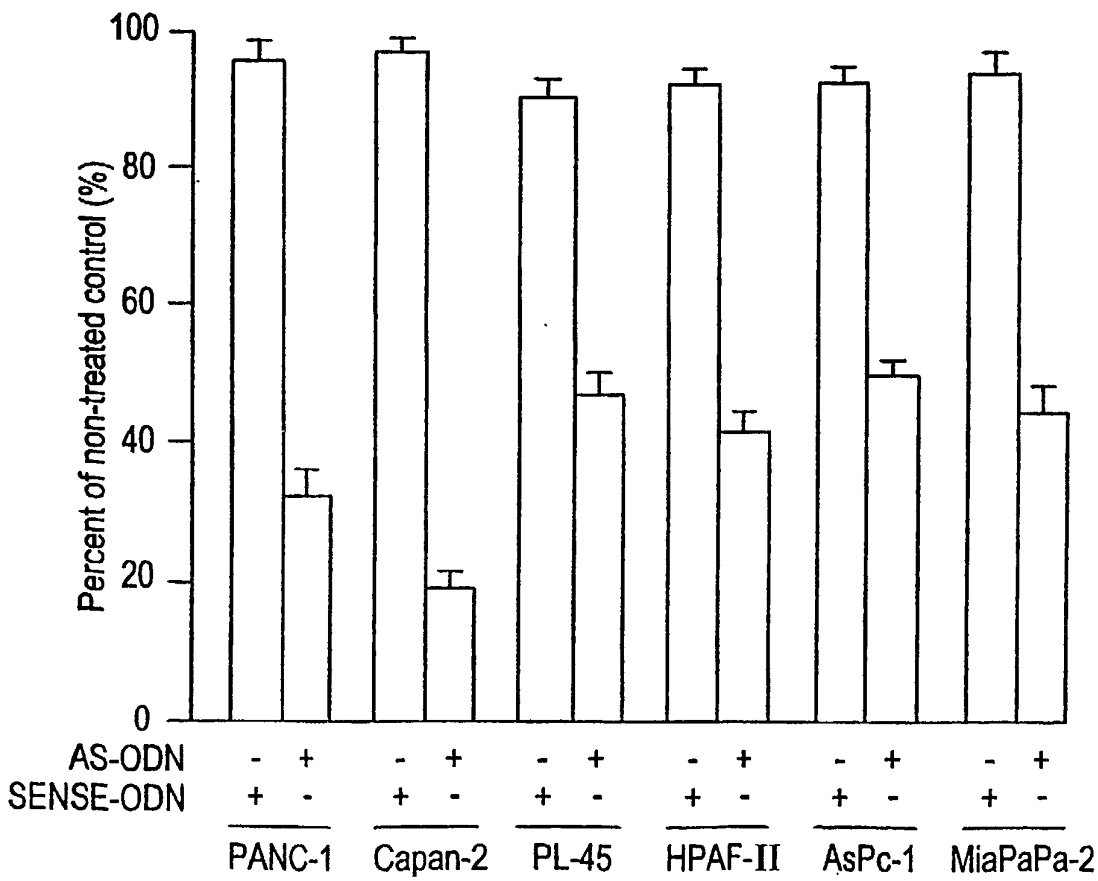
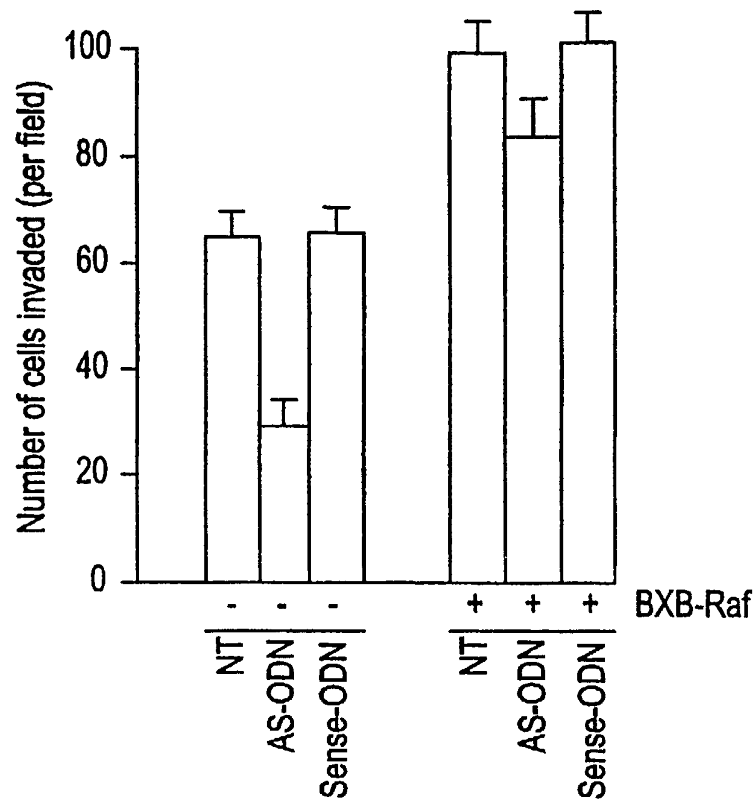
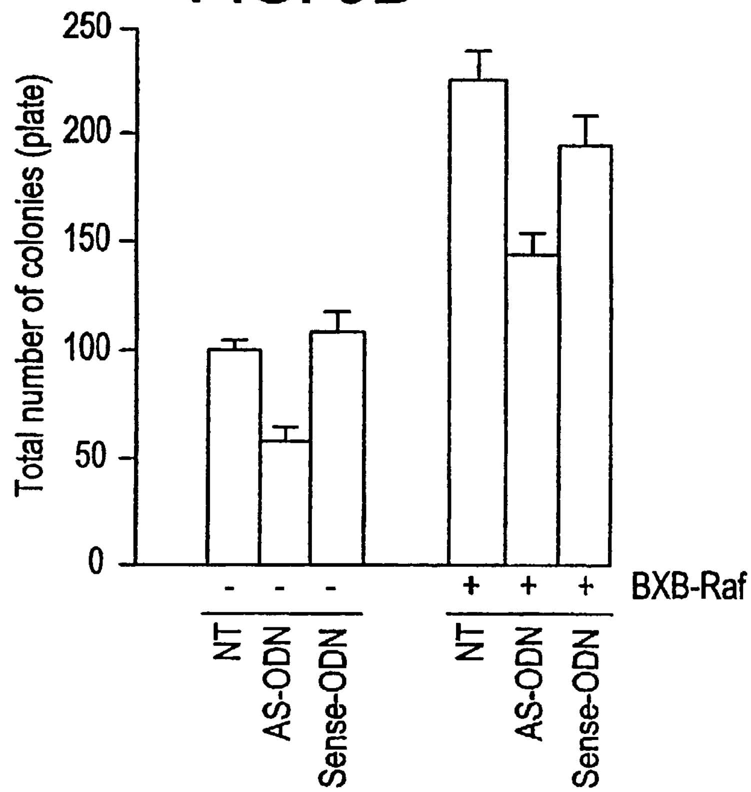

100
80
60
40
20
0
Percent of non-treated control (%)
AS-ODN
SENSE-ODN
- + - + - + - + - + - +
+ - + - + - + - + - + -
PANC-1
Capan-2
PL-45
HPAF-II
AsPc-1
MiaPaPa-2

FIG. 9C



Number of cells invaded (per field)
100
80
60
40
20
0
- - -
+ + +
BXB-Raf
NT
AS-ODN
Sense-ODN
NT
AS-ODN
Sense-ODN

FIG. 9D



Total number of colonies (plate)
250
200
150
100
50
0
- - -
+ + +
BXB-Raf
NT
AS-ODN
Sense-ODN
NT
AS-ODN
Sense-ODN

FIG. 9E

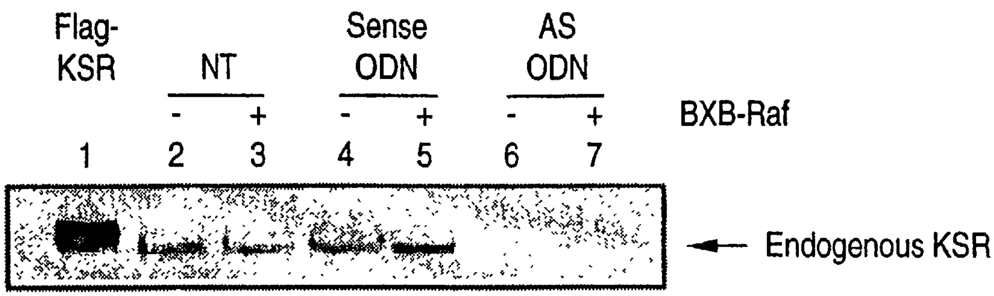

Flag-
KSR
NT
Sense
ODN
AS
ODN
BXB-Raf
- + - + - +
1 2 3 4 5 6 7
Endogenous KSR

FIG. 9F

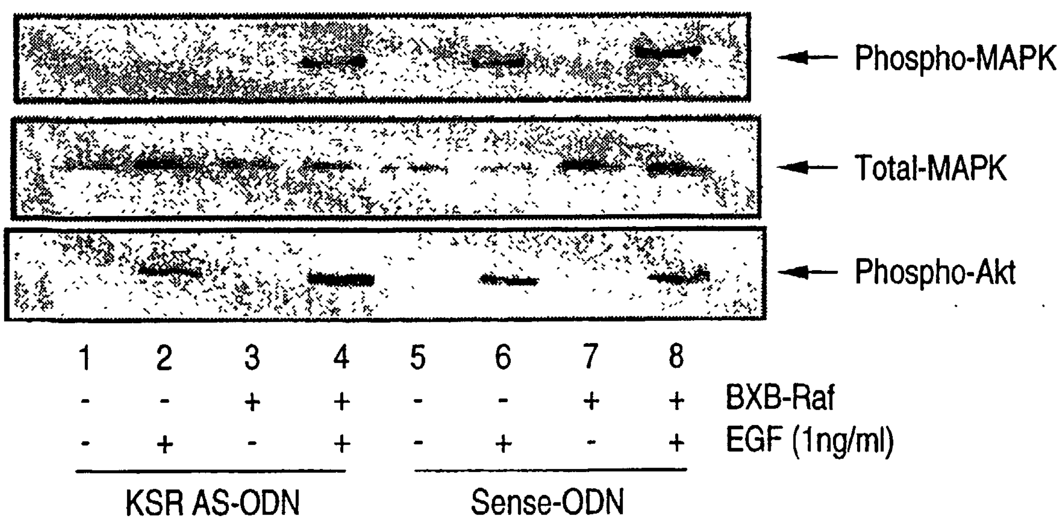

Phospho-MAPK
Total-MAPK
Phospho-Akt
1 2 3 4 5 6 7 8
- - + + - - + + BXB-Raf
- + - + - + - + EGF (1ng/ml)
KSR AS-ODN
Sense-ODN

FIG. 10A

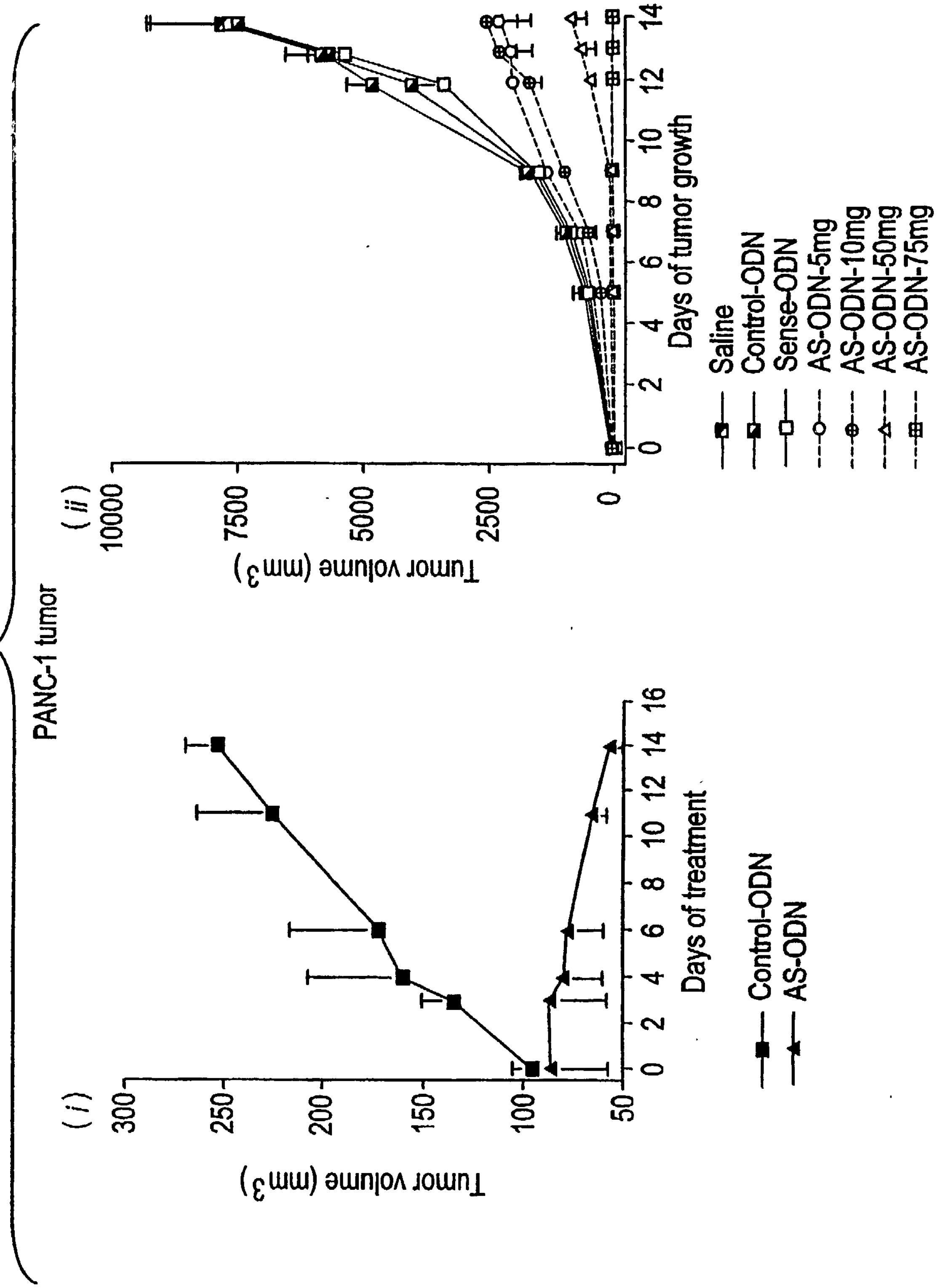

PANC-1 tumor
(i)
Tumor volume (mm3)
300
250
200
150
100
50
0
2
4
6
8
10
12
14
16
Days of treatment
Control-ODN
AS-ODN
(ii)
Tumor volume (mm3)
10000
7500
5000
2500
0
Days of tumor growth
Saline
Control-ODN
Sense-ODN
AS-ODN-5mg
AS-ODN-10mg
AS-ODN-50mg
AS-ODN-75mg

FIG. 10B

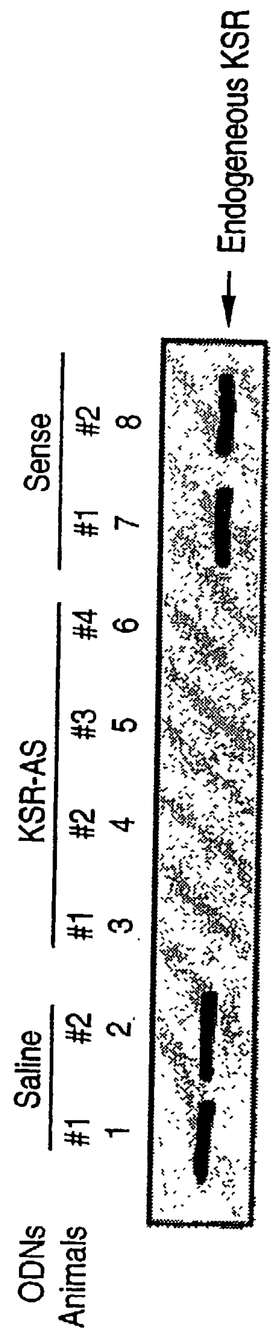


FIG. 10C

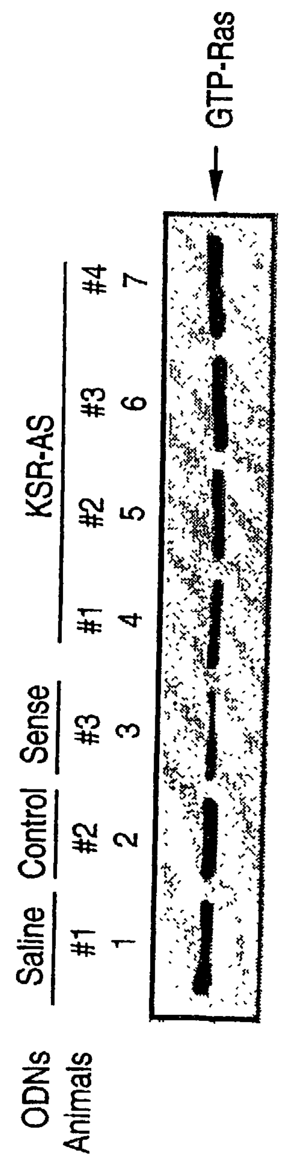
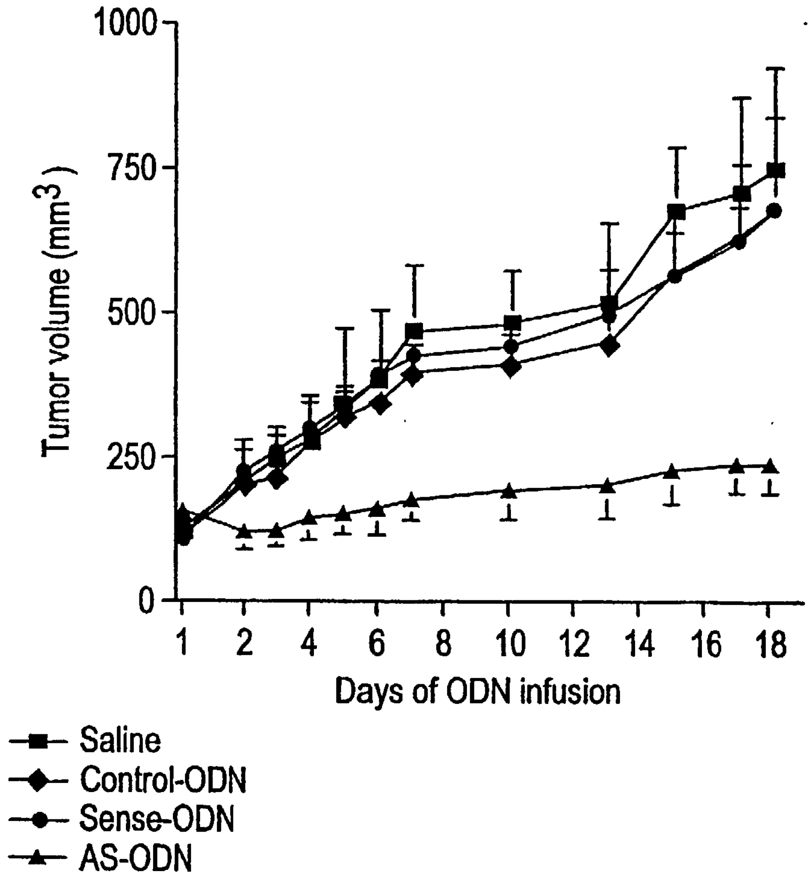


FIG. 10D

A549 tumor

(*i*)



(*ii*)

Number of lung metastases foci
(whole lung surface)

Dose of infusion (mg/ kg /Day)	Sense-ODNs	AS-ODN	% inhibition
10	7.4 ± 1.4	2.5 ± 0.6	65
25	10.2 ± 1.8	1.4 ± 0.5	86

FIG.11-1

```
Human           MGEK-EGGGGGDAAAAEGGAGAAASRALQQCGQLQ     34
Mouse MDRAALRAAA       K          --          V

                               CA1
Human KLIDISIGSLRGLRTKCAVSNDLTQQEIRTLEAKLVRYICKQRQC   79
Mouse                        S                     K       Q S

Human KLSVAPGERTPELNSYPRFSDWLYTFNVRPEVVQEIPRDLTLDAL   124
Mouse       I SD  A                I          QE

Human LEMNEAKVKETLRRCGASGDECGRLQYALTCLRKVTGLGGEHKED   169
Mouse     D     A   M     W    TE    S     Q                      M

Human SSWSSLDARRESGSGPSTDTLSAASLPWPPGSSQLGRAGNSAQGP   214
Mouse   G     I     DS -L   PM  M   S-----------        A  T

Human RSISVSALPASDSPTPSFSEGLSDTCIPLHASGRLTPRALHSFIT   259
Mouse                    V  GL         S          I

                                   CA2
Human PPTTPQLRRHTKLKPPRTPPPPSRKVFQLLPSFPTLTRSKSHESQ   304
MouseA

Human LGNRIDDVSSMRFDLSHGSPQMVRRDIGLSVTHRFSTKSWLSQVC   349
Mouse             TP  K  E  P        L

                              CA3
Human HVCQKSMIFGVKCKHCRLKCHNKCTKEAPACRISFLPLTRLRRTE   394
Mouse N                                         I       A

Human SVPSDINNPVDRAAEPHFGTLPKALTKKEHPPAMNHLDSSSNPSS   439
Mouse                                            _

                                   CA4
Human TTSSTPSSPAPFPTSSNPSSATTPPNPSPGQRDSRFNFPAAYFIH   484
Mouse                L                                 S -------

Human HRQQFIFPDISAFAHAAPLPEAADGTRLDDQPKADVLEAHEAEAE   529
Mouse --------         CSC      SST   S           I     GV

Human EPEAGKSEAEDDED-EVDDLPSSRRPWRGPISRKASQTSVYLQEW   573
Mouse                   ED
```

FIG. 11-2

```
                        I             II
Human DDIPFEQVELGEPIGQGRWGRVHRGRWHGEVAIRLLEMDGHNQDH    618
Mouse

         III             IV                      V
Human LKLFKKEVMNYRQTRHENVVLFMGACMNPPHLAIITSFCKGRTLH    663
Mouse

                                 VIa         VIb
Human SFVRDPKTSLDINKTRQIAQEIIKGMGYLHAKGIVHKDLKSKNVF    708
Mouse

              VII                        VIII
Human YDNGKVVITDFGLFGISGVVREERRENQLKLSHDWLCYLAPEIVR    753
Mouse

                             IX
Human EMTPGKDEDQLPFSKAADVYAFGTVWYELQARDWPLKNQAAEASI    798
Mouse   I  R                             F H P   L
       X                          XI
Human WQIGSGEGMKRVLTSVSLGKEVSEILSACWAFDLQERPSFSLLMD    843
Mouse         VR   A        G

Human MLEKLPKLNRRLSHPGHFWKSAEL                         867
Mouse    R                  DINSSKVMPRFERFGLGTLESGN

Mouse PKM
```

FIG. 12A-1

1 GAATTCCCTC GGGGCTTTCC TGCCGAGGCG CCCGTGTCCC CGGGCTCCTC GCCTCGGCCC
61 CCAGCGGCCC CGATGCCGAG GCATGGATAG AGCGGCGTTG CGCGCGGCAG CGATGGGCGA
121 GAAAAAGGAG GGCGGCGGCG GGGGCGCCGC GGCGGACGGG GGCGCAGGGG CCGCCGTCAG
181 CCGGGCGCTG CAGCAGTGCG GCCAGCTGCA GAAGCTCATC GATATCTCCA TCGGCAGTCT
241 GCGCGGGCTG CGCACCAAGT GCTCAGTGTC TAACGACCTC ACACAGCAGG AGATCCGGAC
301 CCTAGAGGCA AAGCTGGTGA AATACATTTG CAAGCAGCAG CAGAGCAAGC TTAGTGTGAC
361 CCCAAGCGAC AGGACCGCCG AGCTCAACAG CTACCCACGC TTCAGTGACT GGCTGTACAT
421 CTTCAACGTG AGGCCTGAGG TGGTGCAGGA GATCCCCCAA GAGCTCACAC TGGATGCTCT
481 GCTGGAGATG GACGAGGCCA AAGCCAAGGA GATGCTGCGG CGCTGGGGGG CCAGCACGGA
541 GGAGTGCAGC CGCCTACAGC AAGCCCTTAC CTGCCTTCGG AAGGTGACTG GCCTGGGAGG
601 GGAGCACAAA ATGGACTCAG GTTGGAGTTC AACAGATGCT CGAGACAGTA GCTTGGGGCC
661 TCCCATGGAC ATGCTTTCCT CGCTGGGCAG AGCGGGTGCC AGCACTCAGG GACCCCGTTC
721 CATCTCCGTG TCCGCCCTGC CTGCCTCAGA CTCTCCGGTC CCCGGCCTCA GTGAGGGCCT
781 CTCGGACTCC TGTATCCCCT TGCACACCAG CGGCCGGCTG ACCCCCCGGG CCCTGCACAG
841 CTTCATCACG CCCCCTACCA CACCCCAGCT ACGACGGCAC GCCAAGCTGA AGCCACCAAG
901 GACACCCCCA CCGCCAAGCC GCAAGGTCTT CCAGCTGCTC CCCAGCTTCC CCACACTCAC
961 ACGGAGCAAG TCCCACGAGT CCCAGCTGGG AAACCGAATC GACGACGTCA CCCCGATGAA
1021 GTTTGAACTC CCTCATGGAT CCCCACAGCT GGTACGAAGG GATATCGGGC TCTCGGTGAC
1081 GCACAGGTTC TCCACAAAGT CATGGTTGTC ACAGGTGTGC AACGTGTGCC AGAAGAGCAT
1141 GATTTTTGGC GTGAAGTGCA AACACTGCAG GTTAAAATGC CATAACAAGT GCACAAAGGA
1201 AGCTCCCGCC TGCAGGATCA CCTTCCTCCC ACTGGCCAGG CTTCGGAGGA CAGAGTCTGT
1261 CCCGTCAGAT ATCAACAACC CAGTGGACAG AGCAGCAGAG CCCCATTTTG GAACCCTTCC
1321 CAAGGCCCTG ACAAAGAAGG AGCACCCTCC AGCCATGAAC CTGGACTCCA GCAGCAACCC
1381 ATCCTCCACC ACGTCCTCCA CACCCTCATC GCCGGCACCT TTCCTGACCT CATCTAATCC
1441 CTCCAGTGCC ACCACGCCTC CCAACCCGTC ACCTGGCCAG CGGGACAGCA GGTTCAGCTT
1501 CCCAGACATT TCAGCCTGTT CTCAGGCAGC CCCGCTGTCC AGCACAGCCG ACAGTACACG
1561 GCTCGACGAC CAGCCCAAAA CAGATGTGCT AGGTGTTCAC GAAGCAGAGG CTGAGGAGCC
1621 TGAGGCTGGC AAGTCAGAGG CAGAGGATGA CGAGGAGGAT GAGGTGGACG ACCTCCCCAG
1681 CTCCCGCCGG CCCTGGAGGG GCCCCATCTC TCGAAAGGCC AGCCAGACCA GCGTTTACCT
1741 GCAAGAGTGG GACATCCCCT TTGAACAGGT GGAACTGGGC GAGCCCATTG GACAGGGTCG
1801 CTGGGGCCGG GTGCACCGAG GCCGTTGGCA TGGCGAGGTG GCCATTCGGC TGCTGGAGAT
1861 GGACGGCCAC AATCAGGACC ACCTGAAGCT GTTCAAGAAA GAGGTGATGA ACTACCGGCA
1921 GACGCGGCAT GAGAACGTGG TGCTCTTCAT GGGGGCCTGC ATGAACCCAC CTCACCTGGC
1981 CATTATCACC AGCTTCTGCA AGGGGCGGAC ATTGCATTCA TTCGTGAGGG ACCCCAAGAC
2041 GTCTCTGGAC ATCAATAAGA CTAGGCAGAT CGCCCAGGAG ATCATCAAGG GCATGGGTTA
2101 TCTTCATGCA AAAGGCATCG TGCACAAGGA CCTCAAGTCC AAGAATGTCT TCTATGACAA
2161 CGGCAAAGTG GTCATCACAG ACTTCGGGCT GTTTGGGATC TCGGGTGTGG TCCGAGAGGA
2221 ACGGCGCGAG AACCAACTGA AACTGTCACA TGACTGGCTG TGCTACCTGG CCCCCGAGAT
2281 CGTACGAGAA ATGATCCCGG GGCGGGACGA GGACCAGCTG CCCTTCTCCA AAGCAGCCGA
2341 TGTCTATGCA TTCGGGACTG TGTGGTATGA ACTACAGGCA AGAGACTGGC CCTTTAAGCA
2401 CCAGCCTGCT GAGGCCTTGA TCTGGCAGAT TGGAAGTGGG GAAGGAGTAC GGCGCGTCCT
2461 GGCATCCGTC AGCCTGGGGA AGGAAGTCGG CGAGATCCTG TCTGCCTGCT GGGCTTTCGA
2521 TCTGCAGGAG AGACCCAGCT TCAGCCTGCT GATGGACATG CTGGAGAGGC TGCCCAAGCT
2581 GAACCGGCGG CTCTCCCACC CTGGGCACTT TTGGAAGTCG GCTGACATTA ACAGCAGCAA
2641 AGTCATGCCC CGCTTTGAAA GGTTTGGCCT GGGGACCCTG GAGTCCGGTA ATCCAAAGAT

FIG. 12A-2

```
2701 GTAGCCAGCC CTGCACGTTC ATGCAGAGAG TGTCTTCCTT TCGAAAACAT GATCACGAAA
2761 CATGCAGACC ACCACCTCAA GGAATCAGAA GCATTGCATC CCAAGCTGCG GACTGGGAGC
2821 GTGTCTCCTC CCTAAAGGAC GTGCGTGCGT GCGTGCGTGC GTGCGTGCGT GCGTGCGTCA
2881 CCAAGGTGTG TGGAGCTCAG GATCGCAGCC ATACACGCAA CTCCAGATGA TACCACTACC
2941 GCCAGTGTTT ACACAGAGGT TTCTGCCTGG CAAGCTTGGT ATTTTACAGT AGGTGAAGAT
3001 CATTCTGCAG AAGGGTGCTG GCACAGTGGA GCAGCACGGA TGTCCCCAGC CCCCGTTCTG
3061 GAAGACCCTA CAGCTGTGAG AGGCCCAGGG TTGAGCCAGA TGAAAGAAAA GCTGCGTGGG
3121 TGTGGGCTGT ACCCGGAAAA GGGCAGGTGG CAGGAGGTTT GCCTTGGCCT GTGCTTGGGC
3181 CGAGAACCAC ACTAAGGAGC AGCAGCCTGA GTTAGGAATC TATCTGGATT ACGGGGATCA
3241 GAGTTCCTGG AGAGTGGACT CAGTTTCTGC TCTGATCCAG GCCTGTTGTG CTTTTTTTTT
3301 TTCCCCCTTA AAAAAAAAAA AGTACAGACA GAATCTCAGC GGCTTCTAGA CTGATCTGAT
3361 GGATCTTAGC CCGGCTTCTA CTGCGGGGGG GAGGGGGGGA GGGATAGCCA CATATCTGTG
3421 GAGACACCCA CTTCTTTATC TGAGGCCTCC AGGTAGGCAC AAAGGCTGTG GAACTCAGCC
3481 TCTATCATCA GACACCCCCC CCCAATGCCT CATTGACCCC CTTCCCCCAG AGCCAAGGGC
3541 TAGCCCATCG GGTGTGTGTA CAGTAAGTTC TTGGTGAAGG AGAACAGGGA CGTTGGCAGA
3601 AGCAGTTTGC AGTGGCCCTA GCATCTTAAA ACCCATTGTC TGTCACACCA GAAGGTTCTA
3661 GACCTACCAC CACTTCCCTT CCCCATCTCA TGGAAACCTT TTAGCCCATT CTGACCCCTG
3721 TGTGTGCTCT GAGCTCAGAT CGGGTTATGA GACCGCCCAG GCACATCAGT CAGGGAGGCT
3781 CTGATGTGAG CCGCAGACCT CTGTGTTCAT TCCTATGAGC TGGAGGGGCT GGACTGGGTG
3841 GGGTCAGATG TGCTTGGCAG GAACTGTCAG CTGCTGAGCA GGGTGGTCCC TGAGCGGAGG
3901 ATAAGCAGCA TCAGACTCCA CAACCAGAGG AAGAAAGAAA TGGGGATGGA GCGGAGACCC
3961 ACGGGCTGAG TCCCGCTGTG GAGTGGCCTT GCAGCTCCCT CTCAGTTAAA ACTCCCAGTA
4021 AAGCCACAGT TCTCCGAGCA CCCAAGTCTG CTCCAGCCGT CTCTTAAAAC AGGCCACTCT
4081 CTGAGAAGGA ATTC
```

FIG. 12B-1

```
1    GCGAAGCTGG TCCGTTACAT TTGTAAGCAG AGGCAGTGCA AGCTGAGCGT GGCTCCCGGT
61   GAGAGGACCC CAGAGCTCAA CAGCTACCCC CGCTTCAGCG ACTGGCTGTA CACTTTCAAC
121  GTGAGGCCGG AGGTGGTGCA GGAGATCCCC CGAGACCTCA CGCTGGATGC CCTGCTGGAG
181  ATGAATGAGG CCAAGGTGAA GGAGACGCTG CGGCGCTGTG GGGCCAGCGG GGATGAGTGT
241  GGCCGTCTGC AGTATGCCCT CACCTGCCTG CGGAAGGTGA CAGGCCTGGC TTCATCACCC
301  CGCCCACCAC ACCCCAGCTG CGACGGCACA CCAAGCTGAA GCCACCACGG ACGCCCCCCC
361  CACCCAGCCG CAAGGTCTTC CAGCTGCTGC CCAGCTTCCC CACACTCACC CGGAGCAAGT
421  CCCATGAGTC TCAGCTGGGG AACCGCATTG ATGACGTCTC CTCGATGAGG TGAGTTGGGA
481  GCACGTTCCT GCACGTGGCT ATGCTGTGGG GCCTCTCTCA TGAGTCAGAG CGGAGGGAGA
541  CAGCTGTGCC TCTGGAGTCT GCTTTTAATT GTCTGGAAAT GCAGAGATGT CTGGTTTTTG
601  CCTGAGCAAA ATAGGAGTTT ATTTTTGTAC TATCCCGAGC TGGCTAAGGA GAGTCACGTA
661  GCTGTGGGCG GGGTCTTGGG GATGAGGAGG GGTACAGCAG GCAGGGACTA TGCTGAAGTG
721  GAGCTGGCTG TAGGAACCCC AGGGAGGCAC AGGGGGAGCA TGAAGAGGAG CTACACTTCC
781  CTCCCTTAGT GCCCGGGCAG AAACTCCCAG GGCCCTTCAC AGAACCTTGG AGGAACATTC
841  AACACCCCCA TCTCTAGGAC AGCCCCAGCC TTGTCATCCT CCAATTGCTG TGGTAACACG
901  GGGACTGGAG CAGTGAGATT ATTAGGCCTT CAGGGCCAGT GTCTCCATGC AGATCAGATG
961  GAGGCGGTGC TTGGCACATA CACCACCTCA CTGCCCATGC CCCCAGAAGT TGGTGCAGAT
1021 CATAAGGTGG CTTTTGGGGC TAATTGATTG AAGTTCCAAC ATAGTCTGTT TCTCCTAGGC
1081 TGGTAGCTGG CACCTTTGGC CCCATGTGTT TTTTAATTAT TTTTTCTTTT GAGACGAAAT
1141 CTCGCTCTAT CACCCAGGCT GAAGTGCAGT AGTGCAATCT CAGCTCACTG CAGCCTCTGC
1201 CTCCCGGGTT CAAGCAATTC TCCTGCCTCA GCCTCCCGAG TAGCCAGGAT TAAAGGTGCC
1261 TGCCACCACA CATGGCTAAT TTTTGTATTT TTAATAGAGA CGGGGTTTCA CCATGTTAGC
1321 CAGGCTGGTC TCAAACTCCT GACCTCAGGT GATCTTCCTG CCTCAGCCTC CCAAAGTGCT
1381 GGGATTACAG GTGTGAGCCA CTGCGCCCAG TCATGCCCAT GTGTTTTGGT GGTCTTGGCT
1441 GCTGATGGGT GGGGTGAGCC CCAGGAGGAA GTTGGGACAA GTCAACCTCA TGGCAGATGT
1501 GCCAGGGAGA GCTGCGGGTG AGATAGATTG TTCCTATCCC CCTCTCCTTG ATGTGGGAGG
1561 ACTCAGTACC TCCAGCACAC CCTTCTCATG GAGGTTGGTT ATGTGGTACT TGGCCTCAAG
1621 TGAACCAGCA CTTCATGAGT CCAGCTTTGT GCTAGACCAG CACTTGGGAT TGAGGGGGGC
1681 AGTGGCCACC CTCGGGGGAC CTTCTGACTC AGAGGACATG AGATGGCCAC ACTCGAGCAC
1741 TGTGTTCCTG ACCTTTCTGG GTCACAGGTC ACCTTGATGA TTGGATGAAA GTCTTAGATC
1801 TTCTTTCCAG AGAAAAGTCT ACAACATTCT ACTGAACCAG TCCAGAGGGT TCCCGGACCC
1861 CCGAAGCCCA CCCATGGGCT GGCTCTGGGA GGCAATGGCG CTGAGTATGG GGGCATCTCT
1921 CGCATGGATC CCCACAGATG GTACGGAGGG ATATCGGGCT GTCGGTGACG CACAGGTTCT
1981 CCACCAAGTC CTGGCTGTCG CAGGTCTGCC ACGTGTGCCA GAAGAGCATG ATATTTGGAG
2041 TGAAGTGCAA GCATTGCAGG TTGAAGTGTC ACAACAAATG TACCAAAGAA GCCCCTGCCT
2101 GTAGAATATC CTTCCTGCCA CTAACTCGGC TTCGGAGGAC AGAATCTGTC CCCTCGGACA
2161 TCAACAACCC GGTGGACAGA GCAGCCGAAC CCCATTTTGG AACCCTCCCC AAAGCACTGA
2221 CAAAGAAGGA GCACCCTCCG GCCATGAATC ACCTGGACTC CAGCAGCAAC CCTTCCTCCA
2281 CCACCTCCTC CACACCCTCC TCACCGGCGC CCTTCCCGAC ATCATCCAAC CCATCCAGCG
```

FIG. 12B-2

2341 CCACCACGCC CCCCAACCCC TCACCTGGCC AGCGGGACAG CAGGTTCAAC TTCCCAGCTG
2401 CCTACTTCAT TCATCATAGA CAGCAGTTTA TCTTTCCAGA CATTTCAGCC TTTGCACACG
2461 CAGCCCCGCT CCCTGAAGCT GCCGACGGTA CCCGGCTCGA TGACCAGCCG AAAGCAGATG
2521 TGTTGGAAGC TCACGAAGCG GAGGCTGAGG AGCCAGAGGC TGGCAAGTCA GAGGCAGAAG
2581 ACGATGAGGA CGAGGTGGAC GACTTGCCGA GCTCTCGCCG GCCCTGGCGG GGCCCCATCT
2641 CTCGCAAGGC CAGCCAGACC AGCGTGTACC TGCAGGAGTG GGACATCCCC TTCGAGCAGG
2701 TAGAGCTGGG CGAGCCCATC GGGCAGGGCC GCTGGGGCCG GGTGCACCGC GGCCGCTGGC
2761 ATGGCGAGGT GGCCATTCGC CTGCTGGAGA TGGACGGCCA CAACCAGGAC CACCTGAAGC
2821 TCTTCAAGAA AGAGGTGATG AACTACCGGC AGACGCGGCA TGAGAACGTG GTGCTCTTCA
2881 TGGGGGCCTG CATGAACCCG CCCCACCTGG CCATTATCAC CAGCTTCTGC AAGGGGCGGA
2941 CGTTGCACTC GTTTGTGAGG GACCCCAAGA CGTCTCTGGA CATCAACAAG ACGAGGCAAA
3001 TCGCTCAGGA GATCATCAAG GGCATGGGAT ATCTTCATGC CAAGGGCATC GTACACAAAG
3061 ATCTCAAATC TAAGAACGTC TTCTATGACA ACGGCAAGGT GGTCATCACA GACTTCGGGC
3121 TGTTTGGGAT CTCAGGCGTG GTCCGAGAGG GACGGCGTGA GAACCAGCTA AAGCTGTCCC
3181 ACGACTGGCT GTGCTATCTG GCCCCTGAGA TTGTACGCGA GATGACCCCC GGGAAGGACG
3241 AGGATCAGCT GCCATTCTCC AAAGCTGCTG ATGTCTATGC ATTTGGGACT GTTTGGTATG
3301 AGCTGCAAGC AAGAGACTGG CCCTTGAAGA ACCAGGCTGC AGAGGCATCC ATCTGGCAGA
3361 TTGGAAGCGG GGAAGGAATG AAGCGTGTCC TGACTTCTGT CAGCTTGGGG AAGGAAGTCA
3421 GTGAGATCCT GTCGGCCTGC TGGGCTTTCG ACCTGCAGGA GAGACCCAGC TTCAGCCTGC
3481 TGATGGACAT GCTGGAGAAA CTTCCCAAGC TGAACCGGCG GCTCTCCCAC CCTGGACACT
3541 TCTGGAAGTC AGCTGAGTTG TAGGCCTGGC TGCCTTGCAT GCACCAGGGG CTTTCTTCCT
3601 CCTAATCAAC AACTCAGCAC CGTGACTTCT GCTAAAATGC AAAATGAGAT GCGGGCACTA
3661 ACCCAGGGGA TGCCACCTCT GCTGCTCCAG TCGTCTCTCT CGAGGCTACT TCTTTTGCTT
3721 TGTTTTAAAA ACTGGCCCTC TGCCCTCTCC ACGTGGCCTG CATATGCCCA AG

FIG. 13A

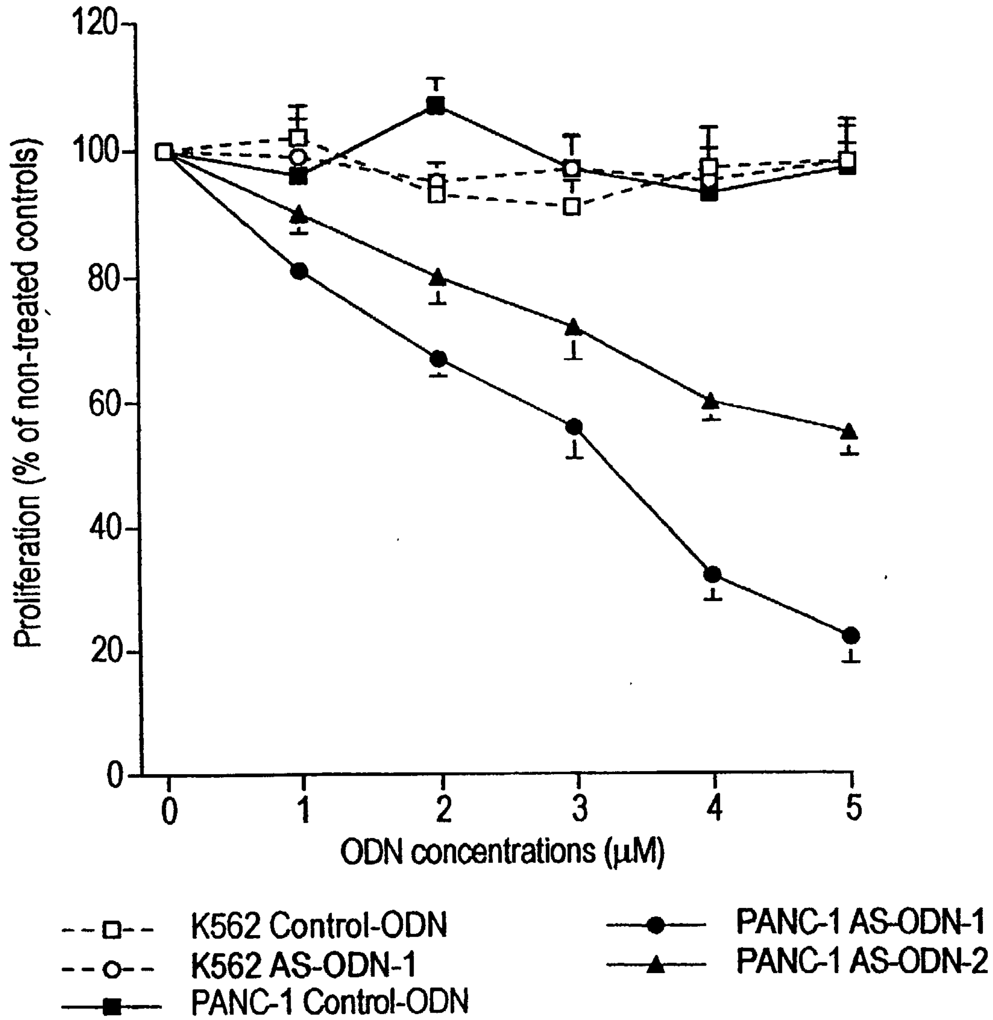


FIG. 13B

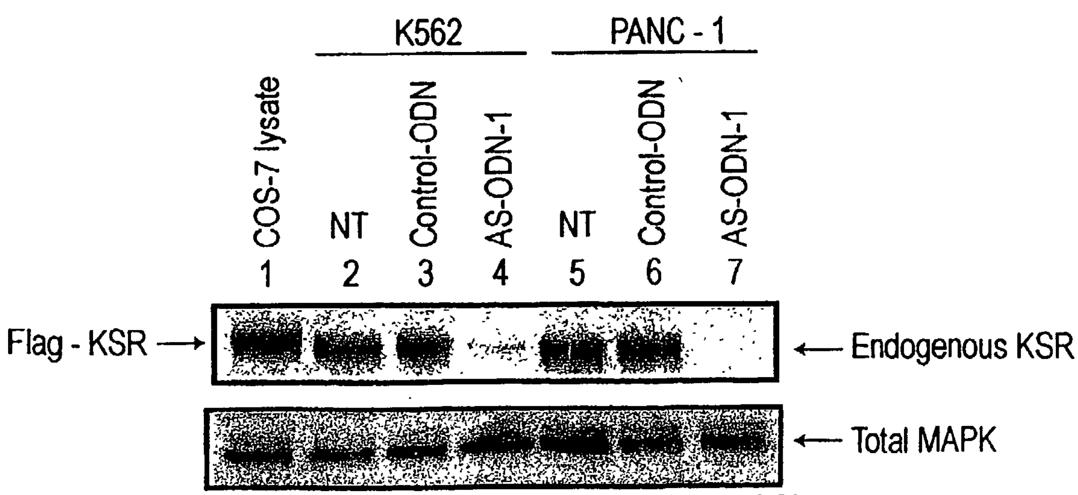


FIG. 14

1	atgggagaga	aggagggcgg	tggcgggggg	gatgcggcgg	ccgcggaggg	tggcgcaggg
60	gccgcggcca	gccgggcgct	gcagcagtgt	gggcagctcc	agaagctcat	cgacatctcc
120	atcggcagtc	tgcgcgggct	gcgcaccaag	tgcgcagtgt	ctaacgacct	cacccagcag
180	gagatacgga	ccctagaggc	aaagctggtc	cgttacattt	gtaagcagag	gcagtgcaag
240	ctgagcgtgg	ctcccggtga	gaggacccca	gagctcaaca	gctacccccg	cttcagcgac
300	tggctgtaca	ctttcaacgt	gaggccggag	gtggtgcagg	agatcccccg	agacctcacg
360	ctggatgccc	tgctggagat	gaatgaggcc	aaggtgaagg	agacgctgcg	gcgctgtggg
420	gccagcgggg	atgagtgtgg	ccgtctgcag	tatgccctca	cctgcctgcg	gaaggtgaca
480	ggcctgggag	gggagcacaa	ggaggactcc	agttggagtt	cattggatgc	gcggcgggaa
540	agtggctcag	ggccttccac	ggacaccctc	tcagcagcca	gcctgccctg	gcccccaggg
600	agctcccagc	tgggcagagc	aggcaacagc	gcccagggcc	cacgctccat	ctccgtgtca
660	gctctgcccg	cctcagactc	ccccaccccc	agcttcagtg	agggcctctc	agacacctgt
720	attcccctgc	acgccagcgg	ccggctgacc	ccccgtgccc	tgcacagctt	catcaccccg
780	cccaccacac	cccagctgcg	acggcacacc	aagctgaagc	caccacggac	gcccccccca
840	cccagccgca	aggtcttcca	gctgctgccc	agcttcccca	cactcacccg	gagcaagtcc
900	catgagtctc	agctggggaa	ccgcattgat	gacgtctcct	cgatgaggtt	tgatctctcg
960	catggatccc	cacagatggt	acggagggat	atcgggctgt	cggtgacgca	caggttctcc
1020	accaagtcct	ggctgtcgca	ggtctgccac	gtgtgccaga	agagcatgat	atttggagtg
1080	aagtgcaagc	attgcaggtt	gaagtgtcac	aacaaatgta	ccaaagaagc	ccctgcctgt
1140	agaatatcct	tcctgccact	aactcggctt	cggaggacag	aatctgtccc	ctcggacatc
1200	aacaacccgg	tggacagagc	agccgaaccc	cattttggaa	ccctccccaa	agcactgaca
1260	aagaaggagc	accctccggc	catgaatcac	ctggactcca	gcagcaaccc	ttcctccacc
1320	acctcctcca	caccctcctc	accggcgccc	ttcccgacat	catccaaccc	atccagcgcc
1380	accacgcccc	ccaacccctc	acctggccag	cgggacagca	ggttcaactt	cccagctgcc
1440	tacttcattc	atcatagaca	gcagtttatc	tttccagaca	tttcagcctt	tgcacacgca
1500	gccccgctcc	ctgaagctgc	cgacggtacc	cggctcgatg	accagccgaa	agcagatgtg
1560	ttggaagctc	acgaagcgga	ggctgaggag	ccagaggctg	gcaagtcaga	ggcagaagac
1620	gatgaggacg	aggtggacga	cttgccgagc	tctcgccggc	cctggcgggg	ccccatctct
1680	cgcaaggcca	gccagaccag	cgtgtacctg	caggagtggg	acatcccctt	cgagcaggta
1740	gagctgggcg	agcccatcgg	gcagggccgc	tggggccggg	tgcaccgcgg	ccgctggcat
1800	ggcgaggtgg	ccattcgcct	gctggagatg	gacggccaca	accaggacca	cctgaagctc
1860	ttcaagaaag	aggtgatgaa	ctaccggcag	acgcggcatg	agaacgtggt	gctcttcatg
1920	ggggcctgca	tgaacccgcc	ccacctggcc	attatcacca	gcttctgcaa	ggggcggacg
1980	ttgcactcgt	ttgtgaggga	ccccaagacg	tctctggaca	tcaacaagac	gaggcaaatc
2040	gctcaggaga	tcatcaaggg	catgggatat	cttcatgcca	agggcatcgt	acacaaagat
2100	ctcaaatcta	agaacgtctt	ctatgacaac	ggcaaggtgg	tcatcacaga	cttcgggctg
2160	tttgggatct	caggcgtggt	ccgagaggga	cggcgtgaga	accagctaaa	gctgtcccac
2220	gactggctgt	gctatctggc	ccctgagatt	gtacgcgaga	tgacccccgg	gaaggacgag
2280	gatcagctgc	cattctccaa	agctgctgat	gtctatgcat	ttgggactgt	ttggtatgag
2340	ctgcaagcaa	gagactggcc	cttgaagaac	caggctgcag	aggcatccat	ctggcagatt
2400	ggaagcgggg	aaggaatgaa	gcgtgtcctg	acttctgtca	gcttggggaa	ggaagtcagt
2460	gagatcctgt	cggcctgctg	ggctttcgac	ctgcaggaga	gacccagctt	cagcctgctg
2520	atggacatgc	tggagaaact	tcccaagctg	aaccggcggc	tctcccaccc	tggacacttc
2580	tggaagtcag	ctgagttgta	g			

FIG. 15A

```
atgggagagaaggagggcggtggcgggggggatgcggcggccgcggagggtggcgcaggg
 M  G  E  K  E  G  G  G  G  G  D  A  A  A  A  E  G  G  A  G  20

gccgcggccagccgggcgctgcagcagtgtgggcagctccagaagctcatcgacatctcc
 A  A  A  S  R  A  L  Q  Q  C  G  Q  L  Q  K  L  I  D  I  S  40
                                    CA1 (32-72)
atcggcagtctgcgcgggctgcgcaccaagtgcgcagtgtctaacgacctcacccagcag
 I  G  S  L  R  G  L  R  T  K  C  A  V  S  N  D  L  T  Q  Q  60
   AS-ODN3(42-47)               AS-ODN2(52-57)
gagatacggaccctagaggcaaagctggtccgttacatttgtaagcagaggcagtgcaag
 E  I  R  T  L  E  A  K  L  V  R  Y  I  C  K  Q  R  Q  C  K  80
   AS-ODN1(63-68)
ctgagcgtggctcccggtgagaggaccccagagctcaacagctacccccgcttcagcgac
 L  S  V  A  P  G  E  R  T  P  E  L  N  S  Y  P  R  F  S  D  100

tggctgtacactttcaacgtgaggccggaggtggtgcaggagatcccccgagacctcacg
 W  L  Y  T  F  N  V  R  P  E  V  V  Q  E  I  P  R  D  L  T  120

ctggatgccctgctggagatgaatgaggccaaggtgaaggagacgctgcggcgctgtggg
 L  D  A  L  L  E  M  N  E  A  K  V  K  E  T  L  R  R  C  G  140

gccagcggggatgagtgtggccgtctgcagtatgccctcacctgcctgcggaaggtgaca
 A  S  G  D  E  C  G  R  L  Q  Y  A  L  T  C  L  R  K  V  T  160

ggcctgggaggggagcacaaggaggactccagttggagttcattggatgcgcggcgggaa
 G  L  G  G  E  H  K  E  D  S  S  W  S  S  L  D  A  R  R  E  180

agtggctcagggccttccacggacaccctctcagcagccagcctgccctggcccccaggg
 S  G  S  G  P  S  T  D  T  L  S  A  A  S  L  P  W  P  P  G  200

agctcccagctgggcagagcaggcaacagcgcccagggcccacgctccatctccgtgtca
 S  S  Q  L  G  R  A  G  N  S  A  Q  G  P  R  S  I  S  V  S  220

gctctgcccgcctcagactcccccacccccagcttcagtgagggcctctcagacacctgt
 A  L  P  A  S  D  S  P  T  P  S  F  S  E  G  L  S  D  T  C  240

attcccctgcacgccagcggccggctgacccccgtgccctgcacagcttcatcaccccg
 I  P  L  H  A  S  G  R  L  T  P  R  A  L  H  S  F  I  T  P  260

cccaccacaccccagctgcgacggcacaccaagctgaagccaccacggacgcccccccca
 P  T  T  P  Q  L  R  R  H  T  K  L  K  P  P  R  T  P  P  P  280

cccagccgcaaggtcttccagctgctgcccagcttccccacactcacccggagcaagtcc
 P  S  R  K  V  F  Q  L  L  P  S  F  P  T  L  T  R  S  K  S  300
   CA2 (277-289)
catgagtctcagctggggaaccgcattgatgacgtctcctcgatgaggtttgatctctcg
 H  E  S  Q  L  G  N  R  I  D  D  V  S  S  M  R  F  D  L  S  320

catggatccccacagatggtacggagggatatcgggctgtcggtgacgcacaggttctcc
 H  G  S  P  Q  M  V  R  R  D  I  G  L  S  V  T  H  R  F  S  340

accaagtcctggctgtcgcaggtctgccacgtgtgccagaagagcatgatatttggagtg
```

FIG. 15B

```
T  K  S  W  L  S  Q  V  C  H  V  C  Q  K  S  M  I  F  G  V   360
                  CA3 (335-380)
aagtgcaagcattgcaggttgaagtgtcacaacaaatgtaccaaagaagcccctgcctgt
K  C  K  H  C  R  L  K  C  H  N  K  C  T  K  E  A  P  A  C   380

agaatatccttcctgccactaactcggcttcggaggacagaatctgtcccctcggacatc
R  I  S  F  L  P  L  T  R  L  R  R  T  E  S  V  P  S  D  I   400

aacaacccggtggacagagcagccgaaccccatttggaaccctccccaaagcactgaca
N  N  P  V  D  R  A  A  E  P  H  F  G  T  L  P  K  A  L  T   420

aagaaggagcaccctccggccatgaatcacctggactccagcagcaacccttcctccacc
K  K  E  H  P  P  A  M  N  H  L  D  S  S  S  N  P  S  S  T   440

acctcctccacaccctcctcaccggcgcccttcccgacatcatccaacccatccagcgcc
T  S  S  T  P  S  S  P  A  P  F  P  T  S  S  N  P  S  S  A   460
                  CA4 (432-498)
accacgccccccaacccctcacctggccagcgggacagcaggttcaacttcccagctgcc
T  T  P  P  N  P  S  P  G  Q  R  D  S  R  F  N  F  P  A  A   480

tacttcattcatcatagacagcagtttatctttccagacatttcagcctttgcacacgca
Y  F  I  H  H  R  Q  Q  F  I  F  P  D  I  S  A  F  A  H  A   500

gccccgctccctgaagctgccgacggtacccggctcgatgaccagccgaaagcagatgtg
A  P  L  P  E  A  A  D  G  T  R  L  D  D  Q  P  K  A  D  V   520

ttggaagctcacgaagcggaggctgaggagccagaggctggcaagtcagaggcagaagac
L  E  A  H  E  A  E  A  E  E  P  E  A  G  K  S  E  A  E  D   540

gatgaggacgaggtggacgacttgccgagctctcgccggccctggcggggccccatctct
D  E  D  E  V  D  D  L  P  S  S  R  R  P  W  R  G  P  I  S   560

cgcaaggccagccagaccagcgtgtacctgcaggagtgggacatccccttcgagcaggta
R  K  A  S  Q  T  S  V  Y  L  Q  E  W  D  I  P  F  E  Q  V   580

gagctgggcgagcccatcgggcagggccgctggggccgggtgcaccgcggccgctggcat
E  L  G  E  P  I  G  Q  G  R  W  G  R  V  H  R  G  R  W  H   600
   CA5 (565-836, consisting of 11 conserved subdomains)
ggcgaggtggccattcgcctgctggagatggacggccacaaccaggaccacctgaagctc
G  E  V  A  I  R  L  L  E  M  D  G  H  N  Q  D  H  L  K  L   620

ttcaagaaagaggtgatgaactaccggcagacgcggcatgagaacgtggtgctcttcatg
F  K  K  E  V  M  N  Y  R  Q  T  R  H  E  N  V  V  L  F  M   640

ggggcctgcatgaacccgccccacctggccattatcaccagcttctgcaagggggcggacg
G  A  C  M  N  P  P  H  L  A  I  I  T  S  F  C  K  G  R  T   660

ttgcactcgtttgtgagggaccccaagacgtctctggacatcaacaagacgaggcaaatc
L  H  S  F  V  R  D  P  K  T  S  L  D  I  N  K  T  R  Q  I   680

gctcaggagatcatcaagggcatgggatatcttcatgccaagggcatcgtacacaaagat
A  Q  E  I  I  K  G  M  G  Y  L  H  A  K  G  I  V  H  K  D   700
```

FIG. 15C

ctcaaatctaagaacgtcttctatgacaacggcaaggtggtcatcacagacttcgggctg
L K S K N V F Y D N G K V V I T D F G L 720

tttgggatctcaggcgtggtccgagagggacggcgtgagaaccagctaaagctgtcccac
F G I S G V V R E G R R E N Q L K L S H 740

gactggctgtgctatctggcccctgagattgtacgcgagatgacccccgggaaggacgag
D W L C Y L A P E I V R E M T P G K D E 760

gatcagctgccattctccaaagctgctgatgtctatgcatttgggactgtttggtatgag
D Q L P F S K A A D V Y A F G T V W Y E 780

ctgcaagcaagagactggcccttgaagaaccaggctgcagaggcatccatctggcagatt
L Q A R D W P L K N Q A A E A S I W Q I 800

ggaagcggggaaggaatgaagcgtgtcctgacttctgtcagcttggggaaggaagtcagt
G S G E G M K R V L T S V S L G K E V S 820

gagatcctgtcggcctgctgggctttcgacctgcaggagagacccagcttcagcctgctg
E I L S A C W A F D L Q E R P S F S L L 840

atggacatgctggagaaacttcccaagctgaaccggcggctctcccaccctggacacttc
M D M L E K L P K L N R R L S H P G H F 860

tggaagtcagctgagttgtag
W K S A E L -

FIG. 16A

Atggatagagcggcgttgcgcgcggcagcgatgggcgagaaaaaggagggcggcggcggg
M D R A A L R A A A M G E K K E G G G G 20

Ggcgccgcggcggacgggggcgcaggggccgccgtcagccgggcgctgcagcagtgcggc
G A A A D G G A G A A V S R A L Q Q C G 40

Cagctgcagaagctcatcgatatctccatcggcagtctgcgcgggctgcgcaccaagtgc
Q **L Q K L I D I S I G S L R G L R T K C** 60
CA1 (42-81) **AS-ODN3(51-56)**

tcagtgtctaacgacctcacacagcaggagatccggaccctagaggcaaagctggtgaaa
S V S N D L T Q Q E I R T L E A K L V K 80
AS-ODN2(61-66) **AS-ODN1(72-77)**

tacatttgcaagcagcagcagagcaagcttagtgtgaccccaagcgacaggaccgccgag
Y I C K Q Q Q S K L S V T P S D R T A E 100

ctcaacagctacccacgcttcagtgactggctgtacatcttcaacgtgaggcctgaggtg
L N S Y P R F S D W L Y I F N V R P E V 120

gtgcaggagatcccccaagagctcacactggatgctctgctggagatggacgaggccaaa
V Q E I P Q E L T L D A L L E M D E A K 140

gccaaggagatgctgcggcgctggggggccagcacggaggagtgcagccgcctacagcaa
A K E M L R R W G A S T E E C S R L Q Q 160

gcccttacctgccttcggaaggtgactggcctgggaggggagcacaaaatggactcaggt
A L T C L R K V T G L G G E H K M D S G 180

tggagttcaacagatgctcgagacagtagcttggggcctcccatggacatgctttcctcg
W S S T D A R D S S L G P P M D M L S S 200

ctgggcagagcgggtgccagcactcagggaccccgttccatctccgtgtccgccctgcct
L G R A G A S T Q G P R S I S V S A L P 220

gcctcagactctccggtccccggcctcagtgagggcctctcggactcctgtatccccttg
A S D S P V P G L S E G L S D S C I P L 240

cacaccagcggccggctgacccccgggccctgcacagcttcatcacgccccctaccaca
H T S G R L T P R A L H S F I T P P T T 260

ccccagctacgacggcacgccaagctgaagccaccaaggacacccccaccgccaagccgc
P Q L R R H A K L K P P R **T P P P P S R** 280
CA2 (274-286)

aaggtcttccagctgctccccagcttccccacactcacacggagcaagtcccacgagtcc
K V F Q L L P S F P T L T R S K S H E S 300

cagctgggaaaccgaatcgacgacgtcaccccgatgaagtttgaactccctcatggatcc
Q L G N R I D D V T P M K F E L P H G S 320

ccacagctggtacgaagggatatcggcctctcggtgacgcacaggttctccacaaagtca
P Q L V R R D I G L S **V T H R F S T K S** 340

FIG. 16B

```
tggttgtcacaggtgtgcaacgtgtgccagaagagcatgatttttggcgtgaagtgcaaa
 W  L  S  Q  V  C  N  V  C  Q  K  S  M  I  F  G  V  K  C  K  360
                    CA3 (331-377)
cactgcaggttaaaatgccataacaagtgcacaaaggaagctcccgcctgcaggatcacc
 H  C  R  L  K  C  H  N  K  C  T  K  E  A  P  A  C  R  I  T  380

ttcctcccactggccaggcttcggaggacagagtctgtcccgtcagatatcaacaaccca
 F  L  P  L  A  R  L  R  R  T  E  S  V  P  S  D  I  N  N  P  400

gtggacagagcagcagagccccattttggaacccttcccaaggccctgacaaagaaggag
 V  D  R  A  A  E  P  H  F  G  T  L  P  K  A  L  T  K  K  E  420

caccctccagccatgaacctggactccagcagcaacccatcctccaccacgtcctccaca
 H  P  P  A  M  N  L  D  S  S  S  N  P  S  S  T  T  S  S  T  440

ccctcatcgccggcacctttcctgacctcatctaatccctccagtgccaccacgcctccc
 P  S  S  P  A  P  F  L  T  S  S  N  P  S  S  A  T  T  P  P  460
                    CA4 (428-480)
aacccgtcacctggccagcgggacagcaggttcagcttcccagacatttcagcctgttct
 N  P  S  P  G  Q  R  D  S  R  F  S  F  P  D  I  S  A  C  S  480

caggcagccccgctgtccagcacagccgacagtacacggctcgacgaccagcccaaaaca
 Q  A  A  P  L  S  S  T  A  D  S  T  R  L  D  D  Q  P  K  T  500

gatgtgctaggtgttcacgaagcagaggctgaggagcctgaggctggcaagtcagaggca
 D  V  L  G  V  H  E  A  E  A  E  E  P  E  A  G  K  S  E  A  520

gaggatgacgaggaggatgaggtggacgacctccccagctcccgccggccctggaggggc
 E  D  D  E  E  D  E  V  D  D  L  P  S  S  R  R  P  W  R  G  540

cccatctctcgaaaggccagccagaccagcgtttacctgcaagagtgggacatcccctttt
 P  I  S  R  K  A  S  Q  T  S  V  Y  L  Q  E  W  D  I  P  F  560

gaacaggtggaactgggcgagcccattggacagggtcgctggggccgggtgcaccgaggc
 E  Q  V  E  L  G  E  P  I  G  Q  G  R  W  G  R  V  H  R  G  580
   CA5 (548-819, consisting of 11 conserved subdomains)
cgttggcatggcgaggtggccattcggctgctggagatggacggccacaatcaggaccac
 R  W  H  G  E  V  A  I  R  L  L  E  M  D  G  H  N  Q  D  H  600

ctgaagctgttcaagaaagaggtgatgaactaccggcagacgcggcatgagaacgtggtg
 L  K  L  F  K  K  E  V  M  N  Y  R  Q  T  R  H  E  N  V  V  620

ctcttcatgggggcctgcatgaacccacctcacctggccattatcaccagcttctgcaag
 L  F  M  G  A  C  M  N  P  P  H  L  A  I  I  T  S  F  C  K  640

gggcggacattgcattcattcgtgagggaccccaagacgtctctggacatcaataagact
 G  R  T  L  H  S  F  V  R  D  P  K  T  S  L  D  I  N  K  T  660

aggcagatcgcccaggagatcatcaagggcatgggttatcttcatgcaaaaggcatcgtg
 R  Q  I  A  Q  E  I  I  K  G  M  G  Y  L  H  A  K  G  I  V  680

cacaaggacctcaagtccaagaatgtcttctatgacaacggcaaagtggtcatcacagac
```

FIG. 16C

```
 H  K  D  L  K  S  K  N  V  F  Y  D  N  G  K  V  V  I  T  D   700

ttcgggctgtttgggatctcgggtgtggtccgagaggaacggcgcgagaaccaactgaaa
 F  G  L  F  G  I  S  G  V  V  R  E  E  R  R  E  N  Q  L  K   720

ctgtcacatgactggctgtgctacctggcccccgagatcgtacgagaaatgatcccgggg
 L  S  H  D  W  L  C  Y  L  A  P  E  I  V  R  E  M  I  P  G   740

cgggacgaggaccagctgcccttctccaaagcagccgatgtctatgcattcgggactgtg
 R  D  E  D  Q  L  P  F  S  K  A  A  D  V  Y  A  F  G  T  V   760

tggtatgaactacaggcaagagactggccctttaagcaccagcctgctgaggccttgatc
 W  Y  E  L  Q  A  R  D  W  P  F  K  H  Q  P  A  E  A  L  I   780

tggcagattggaagtggggaaggagtacggcgcgtcctggcatccgtcagcctggggaag
 W  Q  I  G  S  G  E  G  V  R  R  V  L  A  S  V  S  L  G  K   800

gaagtcggcgagatcctgtctgcctgctgggctttcgatctgcaggagagacccagcttc
 E  V  G  E  I  L  S  A  C  W  A  F  D  L  Q  E  R  P  S  F   820

agcctgctgatggacatgctggagaggctgcccaagctgaaccggcggctctcccaccct
 S  L  L  M  D  M  L  E  R  L  P  K  L  N  R  R  L  S  H  P   840

gggcacttttggaagtcggctgacattaacagcagcaaagtcatgccccgctttgaaagg
 G  H  F  W  K  S  A  D  I  N  S  S  K  V  M  P  R  F  E  R   860

Tttggcctggggaccctggagtccggtaatccaaagatgtag
 F  G  L  G  T  L  E  S  G  N  P  K  M  -                     880
```

FIG. 17A

```
1     atgggagaga aggagggcgg tggcgggggg gatgcggcgg ccgcggaggg tggcgcaggg
        AS-ODN4(1-18)
60    gccgcggcca gccgggcgct gcagcagtgt gggcagctcc agaagctcat cgacatctcc
120   atcggcagtc tgcgcgggct gcgcaccaag tgcgcagtgt ctaacgacct cacccagcag
             AS-ODN3(124-141)                AS-ODN2(154-171)
180   gagatacgga ccctagaggc aaagctggtc cgttacattt gtaagcagag gcagtgcaag
             AS-ODN1(187-204)      AS-ODN5(205-222)
240   ctgagcgtgg ctcccggtga gaggacccca gagctcaaca gctacccccg cttcagcgac
             AS-ODN6(247-264)
300   tggctgtaca ctttcaacgt gaggccggag gtggtgcagg agatccccg agacctcacg
      AS-ODN7(298-315)      AS-ODN8(321-338)      AS-ODN9(351-368)
360   ctggatgccc tgctggagat gaatgaggcc aaggtgaagg agacgctgcg gcgctgtggg
                        AS-ODN10(379-396)
420   gccagcgggg atgagtgtgg ccgtctgcag tatgccctca cctgcctgcg gaaggtgaca
480   ggcctgggag gggagcacaa ggaggactcc agttggagtt cattggatgc gcggcgggaa
                                    AS-ODN11(511-528)     AS-ODN12
540   agtggctcag ggccttccac ggacaccctc tcagcagcca gcctgccctg gccccaggg
      (531-548)
600   agctcccagc tgggcagagc aggcaacagc gcccagggcc cacgctccat ctccgtgtca
660   gctctgcccg cctcagactc ccccaccccc agcttcagtg agggcctctc agacacctgt
720   attcccctgc acgccagcgg ccggctgacc ccccgtgccc tgcacagctt catcaccccg
780   cccaccacac cccagctgcg acggcacacc aagctgaagc caccacggac gcccccccca
840   cccagccgca aggtcttcca gctgctgccc agcttcccca cactcacccg gagcaagtcc
900   catgagtctc agctggggaa ccgcattgat gacgtctcct cgatgaggtt tgatctctcg
960   catggatccc cacagatggt acggagggat atcgggctgt cggtgacgca caggttctcc
1020  accaagtcct ggctgtcgca ggtctgccac gtgtgccaga agagcatgat atttggagtg
1080  aagtgcaagc attgcaggtt gaagtgtcac aacaaatgta ccaaagaagc ccctgcctgt
1140  agaatatcct tcctgccact aactcggctt cggaggacag aatctgtccc ctcggacatc
1200  aacaacccgg tggacagagc agccgaaccc cattttggaa ccctccccaa agcactgaca
1260  aagaaggagc accctccggc catgaatcac ctggactcca gcagcaaccc ttcctccacc
1320  acctcctcca caccctcctc accggcgccc ttcccgacat catccaaccc atccagcgcc
1380  accacgcccc ccaacccctc acctggccag cgggacagca ggttcaactt cccagctgcc
1440  tacttcattc atcatagaca gcagtttatc tttccagaca tttcagcctt tgcacacgca
1500  gccccgctcc ctgaagctgc cgacggtacc cggctcgatg accagccgaa agcagatgtg
1560  ttggaagctc acgaagcgga ggctgaggag ccagaggctg gcaagtcaga ggcagaagac
1620  gatgaggacg aggtggacga cttgccgagc tctcgccggc cctggcgggg ccccatctct
1680  cgcaaggcca gccagaccag cgtgtacctg caggagtggg acatcccctt cgagcaggta
1740  gagctgggcg agcccatcgg gcagggccgc tggggccggg tgcaccgcgg ccgctggcat
1800  ggcgaggtgg ccattcgcct gctggagatg gacggccaca accaggacca cctgaagctc
1860  ttcaagaaag aggtgatgaa ctaccggcag acgcggcatg agaacgtggt gctcttcatg
1920  ggggcctgca tgaacccgcc ccacctggcc attatcacca gcttctgcaa ggggcggacg
1980  ttgcactcgt ttgtgaggga ccccaagacg tctctggaca tcaacaagac gaggcaaatc
2040  gctcaggaga tcatcaaggg catgggatat cttcatgcca agggcatcgt acacaaagat
```

FIG. 17B

2100 ctcaaatcta agaacgtctt ctatgacaac ggcaaggtgg tcatcacaga cttcgggctg
2160 tttgggatct caggcgtggt ccgagaggga cggcgtgaga accagctaaa gctgtcccac
2220 gactggctgt gctatctggc ccctgagatt gtacgcgaga tgacccccgg gaaggacgag
2280 gatcagctgc cattctccaa agctgctgat gtctatgcat ttgggactgt ttggtatgag
2340 ctgcaagcaa gagactggcc cttgaagaac caggctgcag aggcatccat ctggcagatt
2400 ggaagcgggg aaggaatgaa gcgtgtcctg acttctgtca gcttggggaa ggaagtcagt
2460 gagatcctgt cggcctgctg ggctttcgac ctgcaggaga gacccagctt cagcctgctg
2520 atggacatgc tggagaaact tcccaagctg aaccggcggc tctcccaccc tggacacttc
2580 tggaagtcag ctgagttgta g

FIG. 18

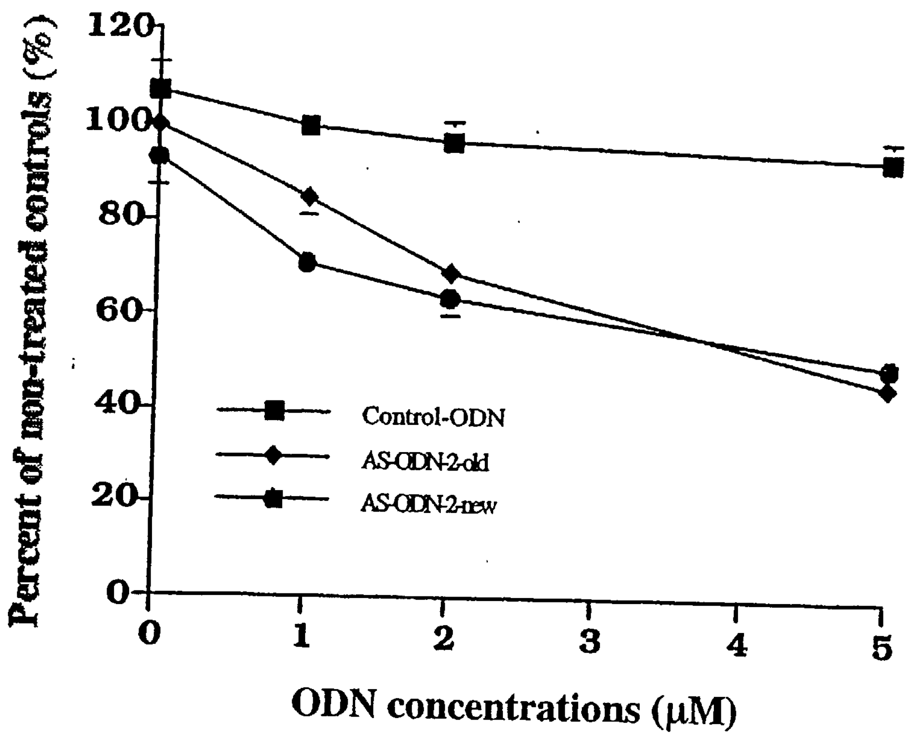

Percent of non-treated controls (%)
120
100
80
60
40
20
0
0
1
2
3
4
5
ODN concentrations (μM)
Control-ODN
AS-ODN-2-old
AS-ODN-2-new

FIG. 19

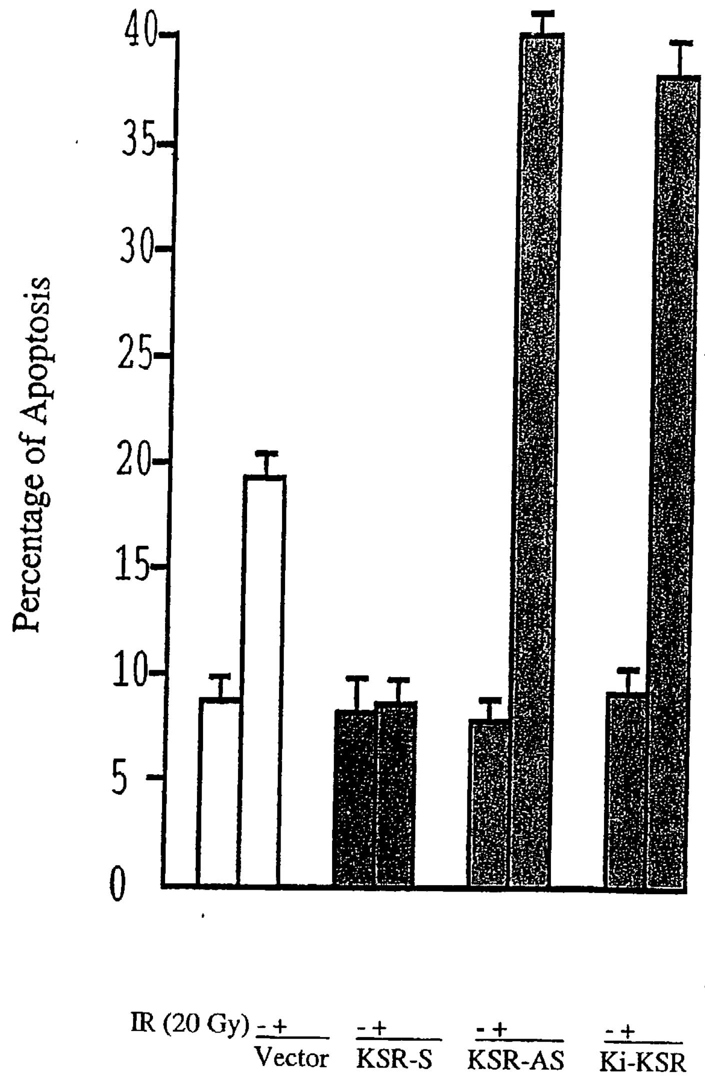

40
35
30
25
20
15
10
5
0
Percentage of Apoptosis
IR (20 Gy)
- +
Vector
- +
KSR-S
- +
KSR-AS
- +
Ki-KSR

FIG. 20

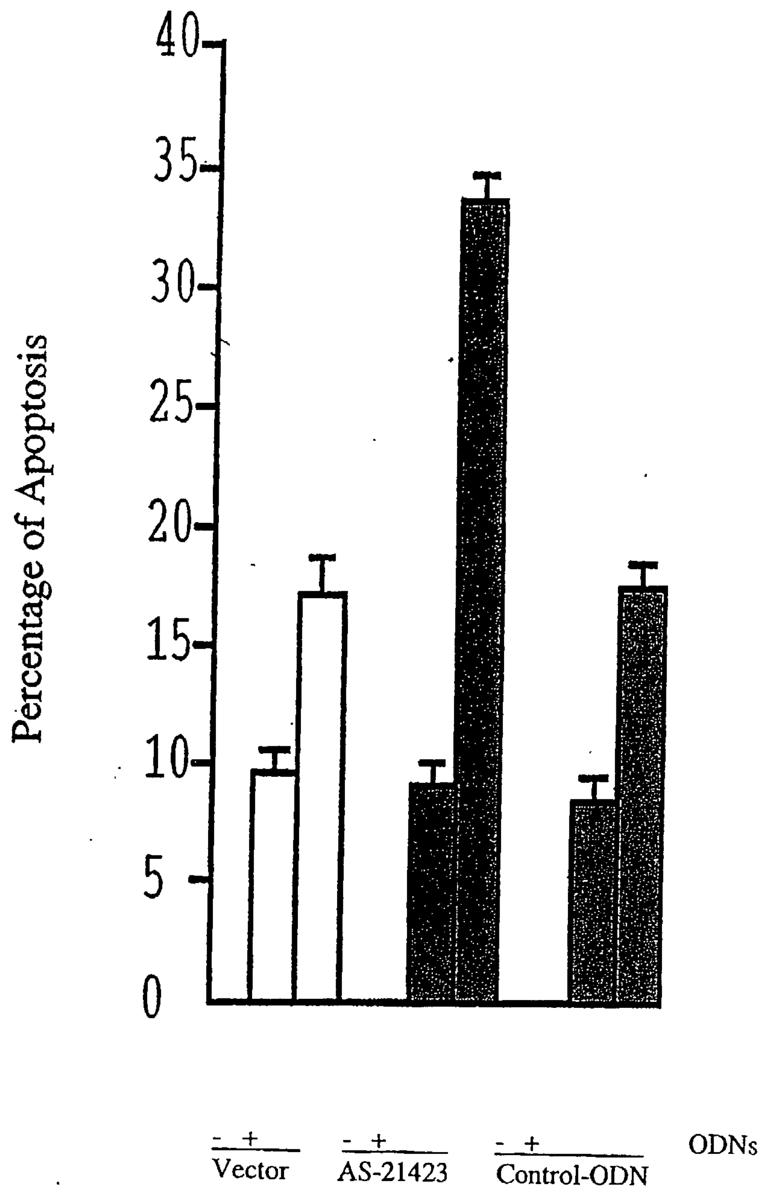
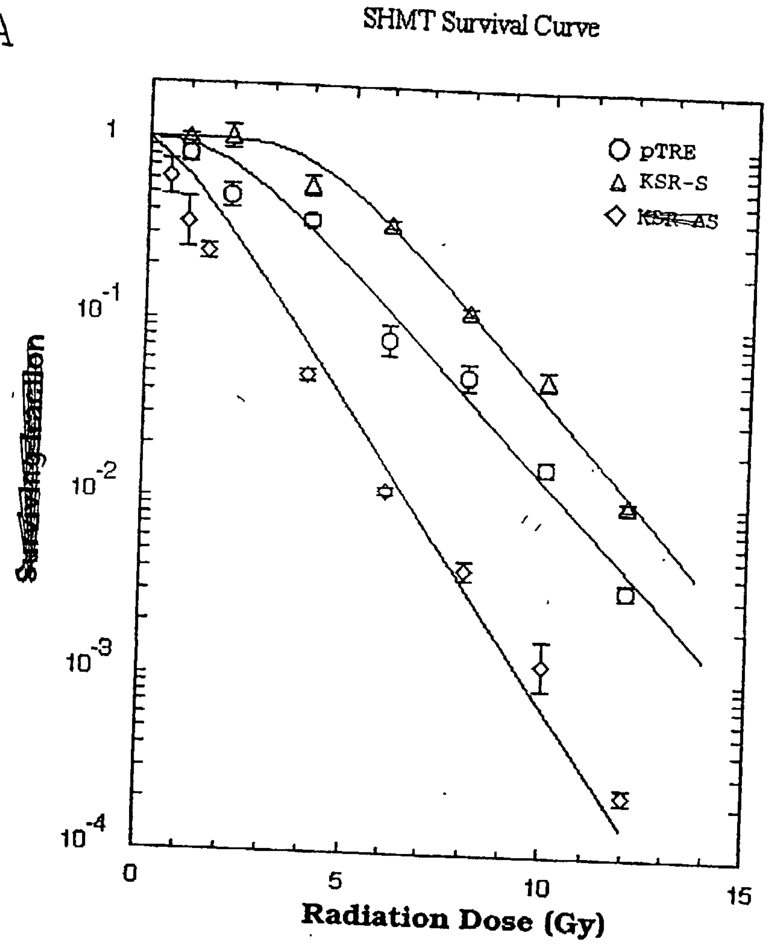

40
35
30
25
20
15
10
5
0
Percentage of Apoptosis
- +
Vector
- +
AS-21423
- +
Control-ODN
ODNs

FIG. 21

A



B

Cell lines	D0 (Gy)	Dq (Gy)	$D_{0.1}$ (Gy)	Dose Ratios (Surv.=0.1)
pTRE	1.77 ± 0.14	2.26 ± 0.44	6.27 ± 0.50	
KSR-S	1.65 ± 0.18	4.57 ± 0.24	8.29 ± 0.45	0.76 ± 0.07
KSR-AS	1.21 ± 0.18	0.62 ± 0.38	2.56 ± 0.49	2.44 ± 0.28

FIG. 22B

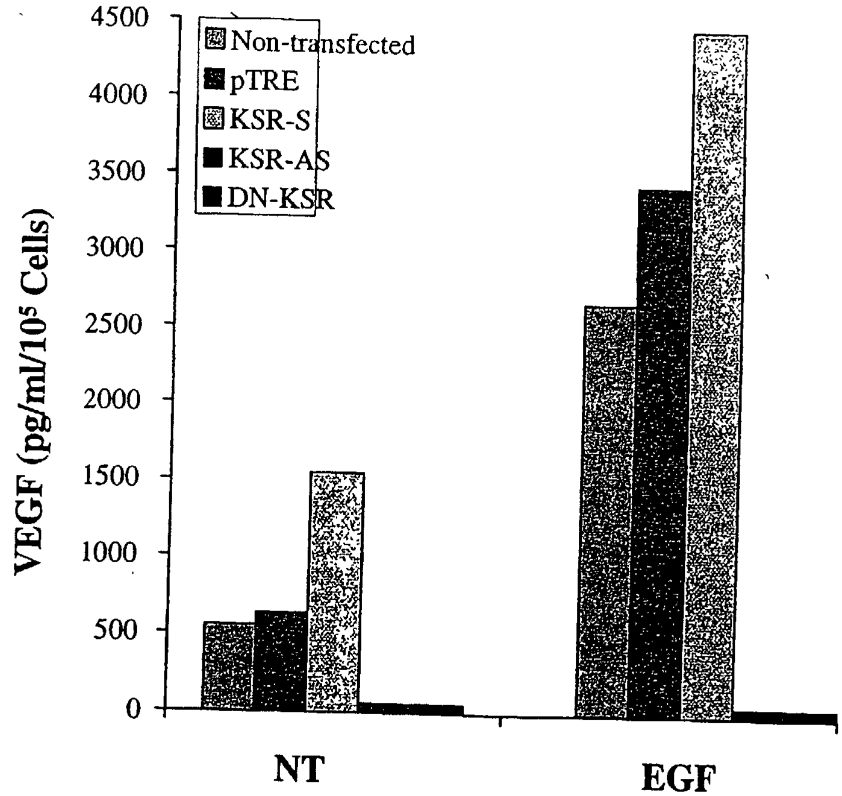
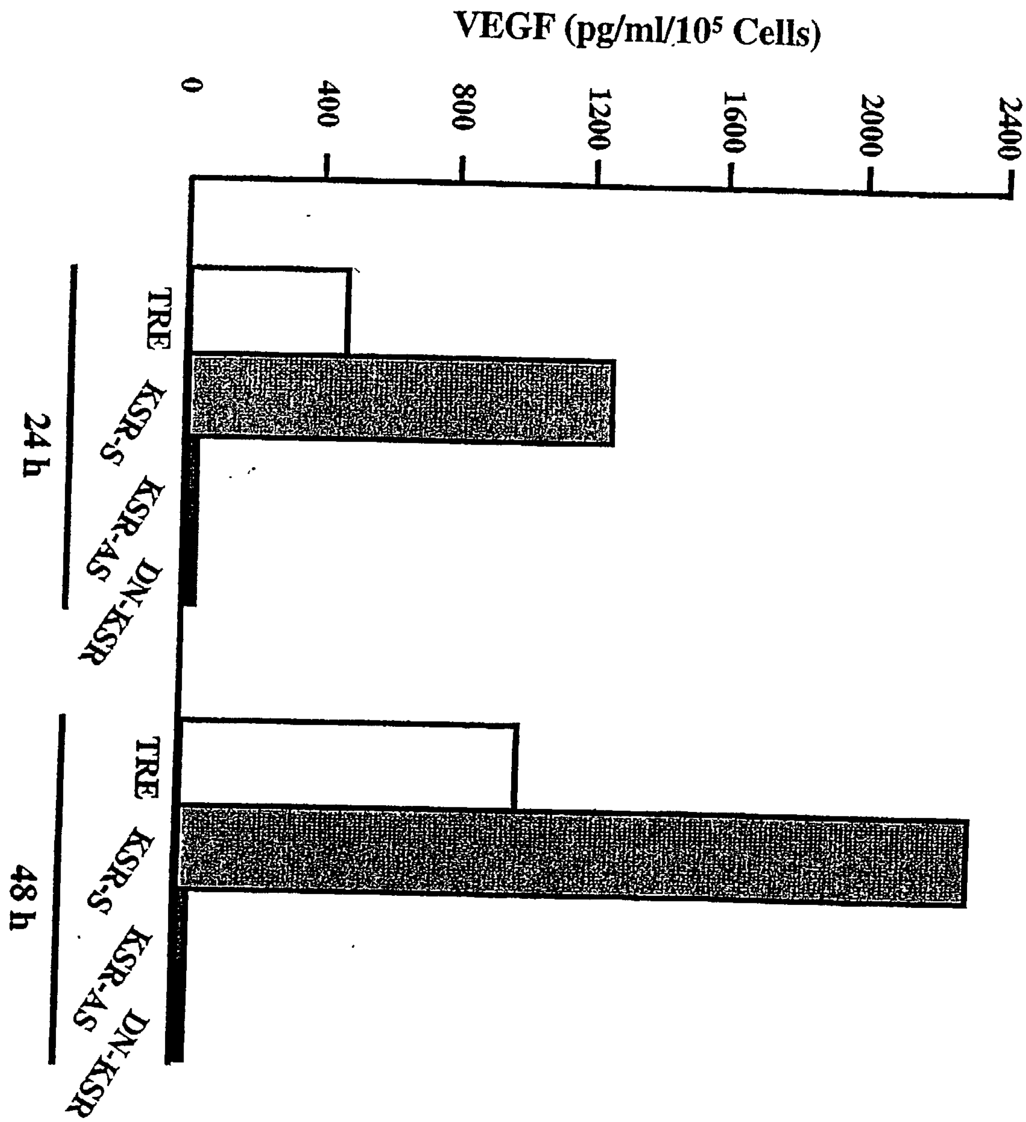

4500
4000
3500
3000
2500
2000
1500
1000
500
0
VEGF (pg/ml/10⁵ Cells)
Non-transfected
pTRE
KSR-S
KSR-AS
DN-KSR
NT
EGF

FIG. 22A


VEGF (pg/ml/10⁵ Cells)
0
400
800
1200
1600
2000
2400
TRE
KSR-S
KSR-AS
DN-KSR
24 h
TRE
KSR-S
KSR-AS
DN-KSR
48 h

SEQUENCE LISTING

<110> Memorial Sloan-Kettering Cancer Center
Kolesnick, Richard N.
Xing, Hong-Mei R.

<120> KINASE SUPPRESSOR OF RAS INACTIVATION FOR THERAPY OF RAS MEDIATED TUMORIGENESIS

<130> 1216-1-006CIPPCT

<140> UNASSIGNED
<141> 2004-12-03

<150> 60/384,228
<151> 2002-05-30

<150> 60/460,023
<151> 2003-04-03

<150> PCT/US03/16961
<151> 2003-05-29

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<151> 2003-12-03

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<213> Homo sapiens

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gtgtctaacg acctcacaca gcaggagatc cggaccctag aggcaaagct ggtgaaatac 120

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<211> 41
<212> PRT
<213> Homo sapiens

<400> 2

Leu Gln Lys Leu Ile Asp Ile Ser Ile Gly Ser Leu Arg Gly Leu Arg
1 5 10 15

Thr Lys Cys Ser Val Ser Asn Asp Leu Thr Gln Gln Glu Ile Arg Thr
20 25 30

Leu Glu Ala Lys Leu Val Lys Tyr Ile

35 40

<210> 3
<211> 19
<212> DNA
<213> Homo sapiens

<400> 3
ggcagtctgc gcgggctgc 19

<210> 4
<211> 18
<212> DNA
<213> Homo sapiens

<400> 4
tcagtgtcta acgacctc 18

<210> 5
<211> 18
<212> DNA
<213> Homo sapiens

<400> 5
cggaccctag aggcaaag 18

<210> 6
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> antisense oligonucleotide

<400> 6
cagcccgcgc agactgccg 19

<210> 7
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> antisense oligonucleotide

<400> 7
gaggtcgtta gacactga 18

<210> 8
<211> 16
<212> DNA

<213> Artificial Sequence

<220>
<223> antisense oligonucleotide

<400> 8
ctttgcctct agggtc 16

<210> 9
<211> 873
<212> PRT
<213> Mus musculus

<400> 9

Met Asp Arg Ala Ala Leu Arg Ala Ala Ala Met Gly Glu Lys Lys Glu
1 5 10 15

Gly Gly Gly Gly Gly Ala Ala Ala Asp Gly Gly Ala Gly Ala Ala Val
20 25 30

Ser Arg Ala Leu Gln Gln Cys Gly Gln Leu Gln Lys Leu Ile Asp Ile
35 40 45

Ser Ile Gly Ser Leu Arg Gly Leu Arg Thr Lys Cys Ser Val Ser Asn
50 55 60

Asp Leu Thr Gln Gln Glu Ile Arg Thr Leu Glu Ala Lys Leu Val Lys
65 70 75 80

Tyr Ile Cys Lys Gln Gln Gln Ser Lys Leu Ser Val Thr Pro Ser Asp
85 90 95

Arg Thr Ala Glu Leu Asn Ser Tyr Pro Arg Phe Ser Asp Trp Leu Tyr
100 105 110

Ile Phe Asn Val Arg Pro Glu Val Val Gln Glu Ile Pro Gln Glu Leu
115 120 125

Thr Leu Asp Ala Leu Leu Glu Met Asp Glu Ala Lys Ala Lys Glu Met
130 135 140

Leu Arg Arg Trp Gly Ala Ser Thr Glu Glu Cys Ser Arg Leu Gln Gln
145 150 155 160

Ala Leu Thr Cys Leu Arg Lys Val Thr Gly Leu Gly Gly Glu His Lys

165 170 175

Met Asp Ser Gly Trp Ser Ser Thr Asp Ala Arg Asp Ser Ser Leu Gly
180 185 190

Pro Pro Met Asp Met Leu Ser Ser Leu Gly Arg Ala Gly Ala Ser Thr
195 200 205

Gln Gly Pro Arg Ser Ile Ser Val Ser Ala Leu Pro Ala Ser Asp Ser
210 215 220

Pro Val Pro Gly Leu Ser Glu Gly Leu Ser Asp Ser Cys Ile Pro Leu
225 230 235 240

His Thr Ser Gly Arg Leu Thr Pro Arg Ala Leu His Ser Phe Ile Thr
245 250 255

Pro Pro Thr Thr Pro Gln Leu Arg Arg His Ala Lys Leu Lys Pro Pro
260 265 270

Arg Thr Pro Pro Pro Pro Ser Arg Lys Val Phe Gln Leu Leu Pro Ser
275 280 285

Phe Pro Thr Leu Thr Arg Ser Lys Ser His Glu Ser Gln Leu Gly Asn
290 295 300

Arg Ile Asp Asp Val Thr Pro Met Lys Phe Glu Leu Pro His Gly Ser
305 310 315 320

Pro Gln Leu Val Arg Arg Asp Ile Gly Leu Ser Val Thr His Arg Phe
325 330 335

Ser Thr Lys Ser Trp Leu Ser Gln Val Cys Asn Val Cys Gln Lys Ser
340 345 350

Met Ile Phe Gly Val Lys Cys Lys His Cys Arg Leu Lys Cys His Asn
355 360 365

Lys Cys Thr Lys Glu Ala Pro Ala Cys Arg Ile Thr Phe Leu Pro Leu
370 375 380

Ala Arg Leu Arg Arg Thr Glu Ser Val Pro Ser Asp Ile Asn Asn Pro
385 390 395 400

```
Val Asp Arg Ala Ala Glu Pro His Phe Gly Thr Leu Pro Lys Ala Leu
                405                 410                 415

Thr Lys Lys Glu His Pro Pro Ala Met Asn Leu Asp Ser Ser Ser Asn
            420                 425                 430

Pro Ser Ser Thr Thr Ser Ser Thr Pro Ser Ser Pro Ala Pro Phe Leu
        435                 440                 445

Thr Ser Ser Asn Pro Ser Ser Ala Thr Thr Pro Pro Asn Pro Ser Pro
    450                 455                 460

Gly Gln Arg Asp Ser Arg Phe Ser Phe Pro Asp Ile Ser Ala Cys Ser
465                 470                 475                 480

Gln Ala Ala Pro Leu Ser Ser Thr Ala Asp Ser Thr Arg Leu Asp Asp
                485                 490                 495

Gln Pro Lys Thr Asp Val Leu Gly Val His Glu Ala Glu Ala Glu Glu
            500                 505                 510

Pro Glu Ala Gly Lys Ser Glu Ala Glu Asp Asp Glu Glu Asp Glu Val
        515                 520                 525

Asp Asp Leu Pro Ser Ser Arg Arg Pro Trp Arg Gly Pro Ile Ser Arg
    530                 535                 540

Lys Ala Ser Gln Thr Ser Val Tyr Leu Gln Glu Trp Asp Ile Pro Phe
545                 550                 555                 560

Glu Gln Val Glu Leu Gly Glu Pro Ile Gly Gln Gly Arg Trp Gly Arg
                565                 570                 575

Val His Arg Gly Arg Trp His Gly Glu Val Ala Ile Arg Leu Leu Glu
            580                 585                 590

Met Asp Gly His Asn Gln Asp His Leu Lys Leu Phe Lys Lys Glu Val
        595                 600                 605

Met Asn Tyr Arg Gln Thr Arg His Glu Asn Val Val Leu Phe Met Gly
    610                 615                 620
```

```
Ala Cys Met Asn Pro Pro His Leu Ala Ile Ile Thr Ser Phe Cys Lys
625                 630                 635                 640

Gly Arg Thr Leu His Ser Phe Val Arg Asp Pro Lys Thr Ser Leu Asp
                645                 650                 655

Ile Asn Lys Thr Arg Gln Ile Ala Gln Glu Ile Ile Lys Gly Met Gly
            660                 665                 670

Tyr Leu His Ala Lys Gly Ile Val His Lys Asp Leu Lys Ser Lys Asn
        675                 680                 685

Val Phe Tyr Asp Asn Gly Lys Val Val Ile Thr Asp Phe Gly Leu Phe
    690                 695                 700

Gly Ile Ser Gly Val Val Arg Glu Glu Arg Arg Glu Asn Gln Leu Lys
705                 710                 715                 720

Leu Ser His Asp Trp Leu Cys Tyr Leu Ala Pro Glu Ile Val Arg Glu
                725                 730                 735

Met Ile Pro Gly Arg Asp Glu Asp Gln Leu Pro Phe Ser Lys Ala Ala
            740                 745                 750

Asp Val Tyr Ala Phe Gly Thr Val Trp Tyr Glu Leu Gln Ala Arg Asp
        755                 760                 765

Trp Pro Phe Lys His Gln Pro Ala Glu Ala Leu Ile Trp Gln Ile Gly
    770                 775                 780

Ser Gly Glu Gly Val Arg Arg Val Leu Ala Ser Val Ser Leu Gly Lys
785                 790                 795                 800

Glu Val Gly Glu Ile Leu Ser Ala Cys Trp Ala Phe Asp Leu Gln Glu
                805                 810                 815

Arg Pro Ser Phe Ser Leu Leu Met Asp Met Leu Glu Arg Leu Pro Lys
            820                 825                 830

Leu Asn Arg Arg Leu Ser His Pro Gly His Phe Trp Lys Ser Ala Asp
        835                 840                 845
```

Ile Asn Ser Ser Lys Val Met Pro Arg Phe Glu Arg Phe Gly Leu Gly
850 855 860

Thr Leu Glu Ser Gly Asn Pro Lys Met
865 870

<210> 10
<211> 866
<212> PRT
<213> Homo sapiens

<400> 10

Met Gly Glu Lys Glu Gly Gly Gly Gly Gly Asp Ala Ala Ala Ala Glu
1 5 10 15

Gly Gly Ala Gly Ala Ala Ala Ser Arg Ala Leu Gln Gln Cys Gly Gln
20 25 30

Leu Gln Lys Leu Ile Asp Ile Ser Ile Gly Ser Leu Arg Gly Leu Arg
35 40 45

Thr Lys Cys Ala Val Ser Asn Asp Leu Thr Gln Gln Glu Ile Arg Thr
50 55 60

Leu Glu Ala Lys Leu Val Arg Tyr Ile Cys Lys Gln Arg Gln Cys Lys
65 70 75 80

Leu Ser Val Ala Pro Gly Glu Arg Thr Pro Glu Leu Asn Ser Tyr Pro
85 90 95

Arg Phe Ser Asp Trp Leu Tyr Thr Phe Asn Val Arg Pro Glu Val Val
100 105 110

Gln Glu Ile Pro Arg Asp Leu Thr Leu Asp Ala Leu Leu Glu Met Asn
115 120 125

Glu Ala Lys Val Lys Glu Thr Leu Arg Arg Cys Gly Ala Ser Gly Asp
130 135 140

Glu Cys Gly Arg Leu Gln Tyr Ala Leu Thr Cys Leu Arg Lys Val Thr
145 150 155 160

Gly Leu Gly Gly Glu His Lys Glu Asp Ser Ser Trp Ser Ser Leu Asp
165 170 175

Ala Arg Arg Glu Ser Gly Ser Gly Pro Ser Thr Asp Thr Leu Ser Ala
180 185 190

Ala Ser Leu Pro Trp Pro Pro Gly Ser Ser Gln Leu Gly Arg Ala Gly
195 200 205

Asn Ser Ala Gln Gly Pro Arg Ser Ile Ser Val Ser Ala Leu Pro Ala
210 215 220

Ser Asp Ser Pro Thr Pro Ser Phe Ser Glu Gly Leu Ser Asp Thr Cys
225 230 235 240

Ile Pro Leu His Ala Ser Gly Arg Leu Thr Pro Arg Ala Leu His Ser
245 250 255

Phe Ile Thr Pro Pro Thr Thr Pro Gln Leu Arg Arg His Thr Lys Leu
260 265 270

Lys Pro Pro Arg Thr Pro Pro Pro Pro Ser Arg Lys Val Phe Gln Leu
275 280 285

Leu Pro Ser Phe Pro Thr Leu Thr Arg Arg Lys Ser His Glu Ser Gln
290 295 300

Leu Gly Asn Arg Ile Asp Asp Val Ser Ser Met Arg Phe Asp Leu Ser
305 310 315 320

His Gly Ser Pro Gln Met Val Arg Arg Asp Ile Gly Leu Ser Val Thr
325 330 335

His Arg Phe Ser Thr Lys Ser Trp Leu Ser Gln Val Cys His Val Cys
340 345 350

Gln Lys Ser Met Ile Phe Gly Val Lys Cys Lys His Cys Arg Leu Lys
355 360 365

Cys His Asn Lys Cys Thr Lys Glu Ala Pro Ala Cys Arg Ile Ser Phe
370 375 380

Leu Pro Leu Thr Arg Leu Arg Arg Thr Glu Ser Val Pro Ser Asp Ile
385 390 395 400

```
Asn Asn Pro Val Asp Arg Ala Ala Glu Pro His Phe Gly Thr Leu Pro
                405                 410                 415

Lys Ala Leu Thr Lys Lys Glu His Pro Pro Ala Met Asn His Leu Asp
            420                 425                 430

Ser Ser Ser Asn Pro Ser Ser Thr Thr Ser Ser Thr Pro Ser Ser Pro
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Ala Pro Phe Pro Thr Ser Ser Asn Pro Ser Ser Ala Thr Thr Pro Pro
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Asn Pro Ser Pro Gly Gln Arg Asp Ser Arg Phe Asn Phe Pro Ala Ala
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Tyr Phe Ile His His Arg Gln Gln Phe Ile Phe Pro Asp Ile Ser Ala
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Asp Asp Gln Pro Lys Ala Asp Val Leu Glu Ala His Glu Ala Glu Ala
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Glu Glu Pro Glu Ala Gly Lys Ser Glu Ala Glu Asp Asp Glu Asp Glu
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Val Asp Asp Leu Pro Ser Ser Arg Arg Pro Trp Arg Gly Pro Ile Ser
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Arg Lys Ala Ser Gln Thr Ser Val Tyr Leu Gln Glu Trp Asp Ile Pro
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Phe Glu Gln Val Glu Leu Gly Glu Pro Ile Gly Gln Gly Arg Trp Gly
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Arg Val His Arg Gly Arg Trp His Gly Glu Val Ala Ile Arg Leu Leu
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Glu Met Asp Gly His Asn Gln Asp His Leu Lys Leu Phe Lys Lys Glu
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Gly Ala Cys Met Asn Pro Pro His Leu Ala Ile Ile Thr Ser Phe Cys
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Lys Gly Arg Thr Leu His Ser Phe Val Arg Asp Pro Lys Thr Ser Leu
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Asp Ile Asn Lys Thr Arg Gln Ile Ala Gln Glu Ile Ile Lys Gly Met
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Gly Tyr Leu His Ala Lys Gly Ile Val His Lys Asp Leu Lys Ser Lys
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Asn Val Phe Tyr Asp Asn Gly Lys Val Val Ile Thr Asp Phe Gly Leu
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Phe Gly Ile Ser Gly Val Val Arg Glu Gly Arg Arg Glu Asn Gln Leu
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Lys Leu Ser His Asp Trp Leu Cys Tyr Leu Ala Pro Glu Ile Val Arg
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Glu Met Thr Pro Gly Lys Asp Glu Asp Gln Leu Pro Phe Ser Lys Ala
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Asp Trp Pro Leu Lys Asn Gln Ala Ala Glu Ala Ser Ile Trp Gln Ile
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Gly Ser Gly Glu Gly Met Lys Arg Val Leu Thr Ser Val Ser Leu Gly
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Lys Glu Val Ser Glu Ile Leu Ser Ala Cys Trp Ala Phe Asp Leu Gln
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Glu Arg Pro Ser Phe Ser Leu Leu Met Asp Met Leu Glu Lys Leu Pro
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