

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
23 February 2006 (23.02.2006)

PCT

(10) International Publication Number
WO 2006/020557 A2

(51) International Patent Classification⁷: Not classified

(21) International Application Number:
PCT/US2005/028100

(22) International Filing Date: 9 August 2005 (09.08.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/600,647 10 August 2004 (10.08.2004) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHODS OF USING OR IDENTIFYING AGENTS THAT INHIBIT CANCER GROWTH

(57) Abstract: The present invention is directed in general to methods of inhibiting the growth of cancer cells or tumors. The present invention is also directed to identifying agents useful for the treatment of cancer. These methods generally involve targeting two genes or their encoded proteins, GPR56 or DNER.

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METHODS OF USING OR IDENTIFYING AGENTS THAT INHIBIT CANCER GROWTH

FIELD OF THE INVENTION

5 The present invention is directed in general to methods of inhibiting the growth of cancer cells or tumors. The present invention is also directed to identifying agents useful for the treatment of cancer. These methods generally involve targeting two genes or their encoded proteins, GPR56 or DNER.

10 BACKGROUND OF THE INVENTION

Despite advancements in diagnosis and treatment, there remains a great need for novel methods of treating cancer and for identifying novel agents that inhibit the growth of cancer cells.

15 Cancers are genetic diseases resulting from multiple disease-causing changes in genes or gene expression involving a variety of biological pathways. It is a major cause of mortality worldwide. More specifically, there is a need to identify targets for treating cancer.

20 GPR56, also known as TM7XN1 (7-Transmembrane Protein with no EGF-Like N-Terminal Domains 1; SEQ ID NO:1; accession # NM_005682; Affymetrix probe ID: 206582_s_at), is an orphan G protein-coupled receptor belonging to GPCR superfamily containing 7 transmembrane domains (TM) and the secretin-like receptor of the GPCR subfamily (Liu et al., *Genomics*, 55:296-305 (1999); Zendman et al., *FEBS Lett.*, 446:292-8 (1999)). The secretin-like polypeptide (ligands) include
25 secretin, calcitonin, parathyroid hormone/parathyroid hormone-related peptides and vasoactive intestinal peptide, all of which activate adenylyl cyclase and the phosphatidyl-inositol-calcium pathway. The secretin-like receptors have their own unique '7TM' signature (Liu et al., *supra*; Zendman et al., *supra*).

30 GPR56 is expressed as a 3kb mRNA in various peripheral tissues, with the highest levels in brain, thyroid gland and heart (Zendman et al, *supra*). GPR56 has recently been indicated in cortical patterning, and mutations in GPR56 were observed in patients with bilateral frontoparietal polymicrogyria (BFPP) that was characterized by disorganized cortical lamination primarily in frontal cortex (Piao et al., *Science*, 303:2033-6 (2004)). In addition, a recent biochemical study demonstrated that GPR56 physically interacts with heterotrimeric G protein subunits, Gαq/11 and Gβ,

through tetraspanins CD9 and CD81, implicating functional specificity of GPR56 (Little et al, *Mol Biol Cell*, 5:2375-87 (2004)).

A second receptor, known as DNER, or Delta/Notch-like epidermal growth factor (EGF)-related receptor (SEQ ID NO:3; Accession #: NM_139072), and also known as HE60, is a newly identified human gene, which is found specifically expressed in the developing and mature central nervous system (Eiraku et al., *J. Biol. Chem.*, 277:25400-07 (2002)). DNER contains a single transmembrane domain at its C-terminal end and is presumed to be a putative cell surface protein. It also contains 10 EGF-like repeats in its extracellular domain and its cytoplasmic carboxy-terminal domain contains a tyrosine-based sorting motif.

DNER has been shown to be targeted to dendrites in neurons. A mouse ortholog of HE60, which shares 90% identity with human HE60, was also identified (BET for brain specific, EGF-like transmembrane protein). BET is highly glycosylated and has been found in patches in dendrites and in the somata of neurons and was implicated in establishment of neuronal network. While very little is known regarding its function in the CNS, there has been no report thus far of any role of HE60 outside the CNS.

There continues to be a tremendous need to identify new receptors as therapeutic targets for cancer. The present invention satisfies this need and provides additional benefits as well.

SUMMARY OF THE INVENTION

The present invention centers on the discovery that knockdown or silencing of GPR56 or DNER in cancer cells, including melanoma cells, results either in the death of the cancer cells or a decrease in their growth. Accordingly, the present invention provides methods for reducing the growth of cancers cells or tumors, such as melanoma. The present invention also provides methods for identifying agents useful for the treatment of cancer, such as melanoma. Such methods based upon the binding of agents to GPR56 or DNER protein, or to the gene or mRNA encoding such protein.

Such methods of reducing growth include applying an agent, such as siRNA or an anti-GPR56 or anti-DNER antibody or a small organic molecule to cancer cells or tumors and reducing their growth.

Such methods of identification include applying an agent to cancerous cells in which GPR56 or DNER protein is expressed, and determining the effect of such agent on the cells. An agent that effectively binds to the protein or its mRNA and thereby

causes a decrease in cancer cell proliferation, or an increase in cell death (apoptosis), or otherwise decreases cancerous growth, will be useful for the treatment of the cancer. Representative agents include antisense oligonucleotides, ribozymes, siRNAs, monoclonal and polyclonal antibodies and small organic molecules.

5

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the DNA sequence of GPR56 (SEQ ID NO:1).

Figure 2 shows the amino acid sequence of the protein encoded by GPR56 (SEQ ID NO:2).

10 Figure 3A shows the results of real-time PCR (RT-PCR) experiments performed to assess the expression levels of GPR56 in several cancer cell lines and normal cell lines.

Figure 3B shows a “virtual northern blot” of GPR56 in normal and tumor tissues.

15 Figure 4A shows that knockdown of GPR56 mRNA by two siRNAs stably transduced into the melanoma cell line A2058 results in a decrease in anchorage-dependent growth.

20 Figures 4B and 4C show that knockdown of GPR56 mRNA by two siRNAs either transiently transduced (Figure 4B) or stably transduced (Figure 4C) into the melanoma cell line M14 results in a decrease in anchorage-dependent growth.

Figure 5A shows that knockdown of GPR56 mRNA by two siRNAs stably transduced into the melanoma cell line A2058 results in a decrease in anchorage-independent growth.

25 Figures 5B and 5C show that knockdown of GPR56 mRNA by two siRNAs either transiently transduced (Figure 5B) or stably transduced (Figure 4C) into the melanoma cell line M14 results in a decrease in anchorage-independent growth.

30 Figures 6A and 6B show that stable transduction of siRNA against GPR56 into either the melanoma cell line A2058 (Figure 6A) or the melanoma cell line M14 (Figure 6B) results in reduction of GPR56 mRNA as measured by real-time PCR (RT-PCR) experiments.

Figure 7 compares the effects of GPR56 knockdown by siRNA on apoptosis (as measured by DNA fragmentation) in three cancer cell lines (HCT116, HeLa, M14) and three non-cancerous cell lines (WI-38, IMR90, HUVEC).

Figure 8 shows the DNA sequence of DNER (SEQ ID NO:3).

Figure 9 shows the amino acid sequence of the protein encoded by DNER (SEQ ID NO:4).

Figure 10 shows the results of real-time PCR (RT-PCR) experiments performed to assess the expression levels of DNER in several cancer cell lines and normal cell lines.

Figures 11A, 11B, 11C and 11D show that knockdown of DNER by siRNA in HOP92, H460, A549 and HOP62 cells, respectively, results in a decrease in anchorage-dependent growth.

Figures 12A, 12B, 12C and 12D show that knockdown of DNER by siRNA in HeLa, H460, A549 and HOP62 cells, respectively, results in a decrease in anchorage-independent growth.

Figures 13A, 13B, 13C and 13D show that transient transfection of siRNA against DNER into HeLa, HOP92, H460 and HOP62 cells, respectively, results in reduction of DNER mRNA as measured by real-time PCR (RT-PCR).

Figure 14 shows expression of GPR56 as determined by Real-time (RT-PCR) in several cell lines. Expression is normalized against GPR56 mRNA level in HeLa.

Figure 15 shows the results of HeLa cells stably transduced with lentiviral hairpin siRNA vectors against GPR56 and control. In Figure 15A, Taqman real-time RT-PCR was used to determine GPR56 mRNA levels. In Figure 15B, 1000 cells of HeLa cells with CNTL, B and C siRNAs were plated into the 96-well soft agar culture, and soft agar growth was scored 7 days later using AlamarBlue staining. The experiment was performed twice with three biological replicates. Error bar indicates standard deviation. In Figure 15C, cells were also plated in regular soft agar culture in the 10 cm dishes and allowed to grow for 27 days before colony numbers were quantified by Qcount.

Figure 16 shows A2058 cells stably transduced with lentiviral hairpin siRNA vectors against GPR56 (B & C) and control. In Figure 16A, Taqman real-time RT-PCR was used to determine GPR56 mRNA levels. In Figure 16B, 1000 cells of A2058 cells with CNTL, B and C siRNAs were plated into the 96-well soft agar culture, and soft agar growth was scored 7 days later using AlamarBlue staining. The experiment was performed twice with three biological replicates. Error bar indicates standard deviation. In Figure 16C, 10000 cells were also plated in regular soft agar

culture in the 10 cm dishes and allowed to grow for 27 days before colony numbers quantified by Qcount.

Figure 17 shows PC3 cells stably transduced with lentiviral hairpin siRNA vectors against GPR56 (B & C) and control. In Figure 17A, Taqman real-time RT-PCR was used to determine GPR56 mRNA levels. In Figure 17B, 1000 cells of PC3 cells with CNTL, B and C siRNAs were plated into the 96-well soft agar culture, and soft agar growth was scored 7 days later using AlamarBlue staining. The experiment was performed twice with three biological replicates. Error bar indicates standard deviation.

Figure 18 shows cells stably transduced with lentiviral hairpin siRNA vectors against GPR56 (B & C) and control. 1000 cells of OVCAR8 (Figure 18A) and OVCAR3 (Figure 18B), were plated into the 96-well soft agar culture, and soft agar growth was scored 7 days later using AlamarBlue staining. The experiment was performed twice with three biological replicates. Error bar indicates standard deviation.

Figure 19 shows cells stably transduced with lentiviral hairpin siRNA vectors against GPR56 (B & C) and control. 1000 cells of AsPC1 (Figure 19A), NCI-H460 (Figure 19B) and M14 (Figure 19C) with CNTL, B and C siRNAs were plated into the 96-well soft agar culture, and soft agar growth was scored 7 days later using AlamarBlue staining. The experiment was performed twice with three biological replicates. Error bar indicates standard deviation.

Figure 20 shows over-expression of GPR56 in A2058 (Figure 20A) and NIH3T3 (Figure 20B) that was achieved by transduction and selection of stable cells containing GPR56 cDNA driven by CMV. In Figure 20A, A2058 cells with either LHCX vector or LHCX-GPR56 cDNA were transduced with lentiviral vectors against either GPR56 or CNTL, stable cells were selected and cell growth was determined by AlamarBlue staining in the 96-well plate. Relative cell growth is normalized to that of the siCNTL. The experiment is performed twice with three biological replicates. Error bar indicates standard deviation. In Figure 20B, NIH3T3 cells with either LHCX or LHCX-GPR56 cDNA were plated in 10-cm dishes so that they were confluent the next day. The cells were allowed to grow for another 7-14 days until foci formation occurred, and the number of foci were quantified by eye counting.

Figures 21 and 22 show the effect of GPR56 siRNA induction on cell growth in vitro and xenograft tumor formation in vivo. PC3-MC6-luc stably transduced with

pTRIP/shGPR56 or pTRIP/siCNTL, were grown in media with or without Doxycycline (1 mg/ml) in T75 flasks. The cells were harvested four days later and the RNA were prepared and determined by real-time RT-PCR and normalized to the cells with control shRNA under non-induced condition (A). At the meantime, the cells were also set up in 96 well plates to determine cell growth by AlamarBlue staining four days post induction (Figure 21A). (As shown in Figure 21B, cell growth was also determined at various time points and was expressed as the ratio of the O.D. values under induced and non-induced conditions. All values were also normalized to those of the cells with control shRNA.

As shown in Figures 22A and 22B, Nude mice (*nu/nu*, Simonsen Laboratories, Inc) were implanted with 5×10^6 pTRIP/shGPR56 (20 total), or pTRIP/siCNTL (18 total) transduced PC3-MC6-luc cells. Silencing was induced in 50% of the animals in each group by addition of DOX to drinking water on the day of implantation (day 0). Mean tumor volumes ($1/2 \times \text{length} \times \text{width}^2$) for each group are shown; error bars represent standard error. Figure 22A: pTRIP/shCNTL; Figure 22B: pTRIP-shGPR56. $P < 0.05$ for induced vs. non-induced for GPR56. As shown in Figures 22C and 22D, nude mice were implanted with PC3-MC6-luc cells as above. On day 16 post-implantation, animals with certain tumor volumes ($>100\text{mm}^3$ for shGPR56, $>150\text{mm}^3$ for shCNTL) were regrouped into two subgroups so that each subgroup had the same number of animals and the average tumor volume in each subgroup is similar. One subgroup was treated with DOX and another was not. Mean tumor volumes ($1/2 \times \text{length} \times \text{width}^2$) for each subgroup are shown; error bars represent standard error. Figure 22C: pTRIP/shmCNTL; Figure 22D: pTRIP/shGPR56. $P < 0.05$ for induced vs. non-induced for GPR56.

Figure 23 shows genes involved in integrin signaling pathway are shown. The genes that are down-regulated in the profiling include ITGA and ITGB.

Figure 24 shows the effect of down-regulating GPR56 on FYN expression, adhesion and apoptotic induction. RNAs from A2058 cells used in Figure 16A were also used to determine FYN expression, and both GPR56 and FYN expression is normalized to those of CNTL cells (Figure 24A). As shown in Figure 24B, 96-well plates were coated with fibronectin at 10 mg/ml at 4°C for three days. 10^4 A2058 cells containing either control or GPR56 siRNA C were seeded onto the wells coated with fibronectin. One hour after seeding, the unattached cells were washed away, and the attached cells were scored using the AlamarBlue staining. The assay was

performed twice each with three biological replicates. The average is presented and the error bar indicates standard deviation. In Figure 24C, 10^4 A2058 cells stably transduced with either GPR56 siRNA (B & C) or the control siRNA were seeded in either methylcellulose media. 16 to 18 hours later, cells were harvested and apoptosis was scored using the DNA fragmentation ELISA assay. In Figure 24D, cells were transduced and seeded into 96-well plates. Four days post transduction, cells were harvested and assayed for apoptosis using the DNA fragmentation ELISA kit.

Figure 25 shows FACS analysis for both A2058 and A2058 cells over-expressing GPR56 (A2058+GPR56) using the GD7 antibody (Figure 25A). FACS analysis was also conducted for A2058 cells expressing siRNAs against GPR56 and the control siRNA using GD7 antibody (Figure 25B).

Figure 26 shows western blot analysis conducted with A2058 cells expressing either GPR56 cDNA (Figure 26A) or GPR56 siRNAs (Figure 26B). Purified GPR56 ECD was used as positive control in A. Deglycosylation was conducted by treating the protein lysates with PNGaseF (Figure 26).

Figure 27 shows immunohistochemistry (IHC) using GPR56 antibody. IHC analysis was conducted with both A2058 and A2058+GPR56 cells with GD7 antibody.

Figure 28 shows the effect of GPR56 antibodies on cell growth. Different dilutions (1:25, 1:50 and 1:100) of GPR56 antibodies (GD7, Figure 28A; GD3, Figure 28B; GD5, Figure 28C) and control antibody (Figure 28D) were added to A2058 cells seeded in the 96-well plates. Cells were allowed to grow four days before the cell growth was scored by AlamarBlue staining. The corresponding pre-immunes were used as nonspecific control.

Figure 29 shows the effect of GPR56 antibody on cells over-expressing GPR56 cDNA. Different dilutions (1:25, 1:50 and 1:100) of GPR56 antibody GD7 and its pre-immune were added to NIH3T3 cells with or without the over-expression of hGPR56 (Figure 29A) or A2058 cells with or without GPR56 over-expression (Figure 29B). Cells were allowed to grow four days before the cell growth was scored by AlamarBlue staining.

Figure 30 shows the effect of GPR56 ECD antigen on the antibody-mediated cell toxicity. Different amount of the purified GPR56 extra-cellular domain (Figure 30A) or a control protein (Figure 30B) were pre-incubated with GD7 antibody for 30

minutes before the complex was added to the cells. Cells were allowed to grow for four days before the growth was scored by AlamarBlue staining.

Figure 31 shows apoptotic induction by GPR56 antibody. Different dilutions of GPR56 antibody (GD7) and its pre-immune was added to tumor cell line A2058 (melanoma, Figure 31A), HRE (renal primary cell line, Figure 31B) and PBMC (blood, Figure 31C). After over-night incubation, apoptosis was determined using the DNA-fragmentation Elisa assay (Roche).

Figure 32 shows the effect of GPR56 on GPR56 protein level. 1e6 A2058-HE26 was seeded in T75, GD7 or its pre-immune was added at 1:100 dilution. Cells were incubated for ~48 hrs. Cells were lysed in 1ml lysis buffer (1% Triton X-100/PBS). 10 ul of lysates were loaded for Western blotting using GD7 antibody. Actin antibody was used as the loading control.

Figure 33 shows HE60 (DNER) expression in Hela and HelaHF cell pair. mRNA expression levels of HE60 in the Hela (transformed) and HF (non transformed revertant) was analyzed by Affymetrix GeneChip DNA microarray (U138 A-chip) and Taqman. HE60 mRNA was shown to be down-regulated in HelaHF cells by both analyses.

Figures 34 to 37 show lentiviral shRNA vectors (pSD31) against HE60 (DNER) being used to transduce non-small cell lung cancer (NSCL) Hop62 cells (Figures 34A-C), (NSCL) H460 cells (Figures 35A-C), melanoma line A2058 cells (Figures 36A-C) and prostate cancer PC3 cells (Figure 37A-C). The stable cell lines were generated by selection using puromycin. After selection the cells were plated in either anchorage dependent (liquid culture; Figures 34A, 35A, 36A and 37A) or anchorage independent (soft agar; Figures 34B, 35B, 36B and 37B) culture conditions. Cell growth was measured by alamar blue staining after one week (soft agar) or 3 days (liquid culture). In parallel Taqman analysis was performed on the same cells to evaluate the extent of HE60 mRNA knockdown (Figures 34C, 35C, 36C and 37C).

Figure 38 shows the effect of HE60 (DNER) silencing on the tumorigenicity of cancer cells in a prostate cancer xenograft model. PC3 prostate cancer cells transduced with either lentiviral vectors pSD31-shHE60 or pSD31-shCNTL vector were injected into nude mice. The ability of these cells to form tumors was monitored. Tumor measurements were made on a weekly basis. The y-axis is tumor volume (mm^3).

DETAILED DESCRIPTION OF INVENTION

The present invention centers upon the discovery that knockdown of GPR56 in cancer cells, including melanoma cells, results in inhibition of the cancer. In this regard, "inhibition" means any one or more of the following: a) a decrease in cancer cell division (proliferation); b) an increase in the sensitivity of the cells to undergo apoptosis; or c) an increase in cell death (apoptosis). Accordingly, the present invention provides methods for inhibition of cancers cells or tumors, such as melanoma by administering a compound of the invention, as described below. Such compounds can bind to GPR56 (SEQ ID NO:1), its encoded protein (SEQ ID NO:2), or any of the domains thereof, as described below. The present invention also provides methods of identifying an agent useful for the treatment of cancer, such as melanoma, by applying the agent to the cancerous cells, where it binds to GPR56 protein (SEQ ID NO:2), or to the gene or the mRNA encoding GPR56 protein (SEQ ID NO:1), resulting in the inhibition of the cancer.

It should be noted that all aspect of the subject invention covering SEQ ID NO:2, also cover this sequence where His (H) replaces Gln (Q) at position 306. The invention also covers all amino acid sequences comprising or consisting of positions 1 to 398. These positions include the signal sequence and the first extracellular domain. The invention further includes all of these sequences when beginning at position 26, positions 25-26 being the cleavage point for the mature protein. The invention further covers all nucleic acid sequences that encode the above-described amino acid sequences.

The domains of SEQ ID NO:2 include amino acid 1 to amino acid 26 of SEQ ID NO:2, amino acid 342 to amino acid 394 of SEQ ID NO:2, amino acid 405 to amino acid 427 of SEQ ID NO:2, amino acid 447 to amino acid 469 of SEQ ID NO:2, amino acid 479 to amino acid 501 of SEQ ID NO:2, amino acid 514 to amino acid 536 of SEQ ID NO:2, amino acid 575 to amino acid 597 of SEQ ID NO:2, amino acid 609 to amino acid 631 of SEQ ID NO:2, and amino acid 637 to amino acid 659 of SEQ ID NO:2.

Measurement of cancer cell inhibition can be done, for example, by means of an apoptosis assay, where an increase in the level of apoptosis indicates that the agent introduced into the cell system inhibits the cancer cells. Measurement of inhibition

can also be made by means of an assay that measures cell proliferation, where a decrease in the rate of cell division indicates that the agent inhibits the cancer cells.

Regarding compounds of the invention that can inhibit cancer by binding to GPR56, an example of such a compound is siRNA. Both transient and stable
5 transduction of two separate siRNA molecules (SEQ ID NOS:5 and 6) into several types of cancer cells (melanoma, cervical cancer, HeLa, colon cancer, prostate cancer, lung cancer, breast cancer, ovarian cancer and glioma) targeting GPR56 caused either reduction anchorage dependent (liquid culture) growth or anchorage-independent (soft-agar) growth of the cancerous cells or both types of reduction (see Example 1,
10 parts 3 to 5; and Figures 4, 5 and 16 to 19). Moreover, the transduction of melanoma cells with siRNA against GPR56 resulted in a significant reduction in GPR56 mRNA (see Figure 6) and a significant increase in cell death, as measured by an apoptosis assay (see Figure 7).

By over-expressing GPR56 and examining the phenotype by counting foci,
15 GPR56 is clearly an oncogene. Moreover, over-expression of GPR56 was found in breast, colon, lung, ovarian, pancreatic, skin and CNS cancers. See Example 1, part 6 and Figures 20 and 21. Using a xenograft model, SEQ ID NOS: 7 and 8 were able to regress tumors. See Example 1, part 7 and Figures 21 and 22.

Another molecule of the invention that inhibits cancer is an antibody,
20 specifically an anti-GPR56 antibody. Examples of such antibodies include GD3 to GD8. These antibodies were raised against specific peptides or domains of SEQ ID NO:2 or mixtures thereof. See Table 2, below. Indeed, GD3, 5 and 7 reduced cancer cell growth. See Example 1, parts 12 and 13; and Figures 28, 30 and Table 3.

Moreover, over-expression of GPR56 sensitizes cell growth to antibody treatment.

25 See Figure 29. This shows that the antibodies of the invention can inhibit cancer cells differentially.

An additional focus of the present invention is the discovery that knockdown of DNER in various cancer cells, including lung and cervical cancer, results in inhibition of the cancer. In this regard, "inhibition" means any one or more of the
30 following: a) a decrease in cancer cell division (proliferation); b) an increase in the sensitivity of the cells to undergo apoptosis; or c) an increase in cell death (apoptosis). Accordingly, the present invention provides a method of identifying an agent useful for the treatment of cancer, such as lung and cervical cancer, by applying the agent to the cancerous cells, where it binds to DNER protein (SEQ ID NO:4), or to the gene or

the mRNA encoding DNER protein (SEQ ID NO:3), resulting in the inhibition of the cancer.

Thus, the invention further provides a method of inhibiting cancer cells or tumors, such as melanoma by administering a compound of the invention, as described below. Such compounds can bind to DNER (SEQ ID NO:3), its encoded protein (SEQ ID NO:4), or any of the domains thereof, as described below. The invention further provides a method for identifying an agent that inhibits cancer by applying to the cancerous cells an agent which binds to any domain of DNER protein (SEQ ID NO:4). In particular, these domains are: amino acid 1 to amino acid 29 of SEQ ID NO:4, amino acid 47 or 48 to amino acid 92 of SEQ ID NO:4, amino acid 96 to amino acid 133 of SEQ ID NO:4, amino acid 306 to amino acid 348 of SEQ ID NO:4, amino acid 349 to amino acid 390 of SEQ ID NO:4, amino acid 392 to amino acid 428 of SEQ ID NO:4, amino acid 430 to amino acid 466 of SEQ ID NO:4, amino acid 469 to amino acid 503 of SEQ ID NO:4, amino acid 505 to amino acid 541 of SEQ ID NO:4, amino acid 544 to amino acid 579 of SEQ ID NO:4, amino acid 546 to amino acid 568 of SEQ ID NO:4, amino acid 581 to amino acid 617 of SEQ ID NO:4, and amino acid 639 to amino acid 661 of SEQ ID NO:4. See Example 2, part 1.

Measurement of cancer cell inhibition can be done, for example, by means of an apoptosis assay, where an increase in the level of apoptosis indicates that the agent introduced into the cell system inhibits the cancer cells. Measurement of inhibition can also be made by means of an assay that measures cell proliferation, where a decrease in the rate of cell division indicates that the agent inhibits the cancer cells.

An example of such a molecule that can bind to DNER and inhibit cancer is an siRNA. Transient transfection into: a) four different lung cancer cells lines (HOP92, H460, A549 and HOP62); b) the cervical cancer cells line HeLa; c) the prostate cell line PC-3; and d) the melanoma cancer cell line A2058; (SEQ ID NOS:10 and 12) targeting DNER caused reduction of either anchorage dependent (liquid culture) growth or anchorage-independent (soft-agar) growth of the cancerous cells or both types of reduction (see Example 2, parts 4 and 5; Figures 11, 12 and 34 to 37 and Table 5 below). Moreover, the transfection of these cells with siRNA against DNER resulted in a significant reduction in DNER mRNA (see Figure 13). Finally, using a xenograft model, SEQ ID NOS: 10 and 12 were able to regress tumors. See Example 2, part 6; and Figure 38.

In addition to siRNA constructs, a molecule of the invention (for targeting GPR56 or DNER) also includes ribozymes, antisense molecules, antibodies (as described above and in Examples 1 and 2) and small organic molecules that target or bind to one of the proteins of the invention.

5 An example of an apoptosis assay useful for the practice of the present invention is the Annexin-V binding assay. This assay is based on the relocation of phosphatidylserine to the outer cell membrane. Viable cells maintain an asymmetric distribution of different phospholipids between the inner and outer leaflets of the plasma membrane. Choline-containing phospholipids such as phosphatidylcholine and sphingomyelin are primarily located on the outer leaflet of viable cells and
10 aminophospholipids such as phosphatidylethanolamine and phosphatidylserine (PS) are found at the cytoplasmic (inner) face of viable cells. The distribution of phospholipids in the plasma membrane changes during apoptosis. In particular, PS relocates from the cytoplasmic face to the outer leaflet so called PS exposure. The extent of PS exposure can distinguish apoptotic cells from the non-apoptotic cells.
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Annexin-V is a 35-36 kDa calcium-dependent phospholipid binding protein with high affinity for PS ($K_d \sim 5 \times 10^{-10}$ M). When labeled with a fluorescent dye, Annexin-V can be used as a sensitive probe for PS exposure on the outer leaflet of the cell membrane. The binding of Annexin-V conjugates such as Annexin-V FITC to
20 cells permits differentiation of apoptotic cells (Annexin-V positive) from non-apoptotic cells (Annexin-V negative). Annexin-V binding is observed under two conditions. The first condition is observed in cells midway through the apoptosis pathway. Phosphatidylserine translocates to the outer leaflet of the cell membrane. The second condition is observed in very late apoptosis or when the cells become
25 necrotic and membrane permeabilization occurs. This membrane permeabilization allows Annexin-V to enter cells and bind to phosphatidylserine on the cytoplasmic face of the membrane. Since other causes besides apoptosis can result in necrosis, it is important to distinguish between necrotic and apoptotic cells. Membrane permeabilization also permits entry of other materials to the interior of the cell,
30 including the fluorescent DNA-binding dye propidium iodide. Utilizing dual staining methodology, apoptotic populations can be distinguished from necrotic populations. For example, using the Annexin V-propidium iodide (PI) double staining regime, three populations of cells are distinguishable in twocolor flow cytometry. See

Boersma, et al., Cytometry, 24:123-130 (1996); Martin, et al., J. Exp. Med., 182:1545-1556 (1995).

Another example of an apoptosis assay is the caspase 3/7 assay. Briefly, caspases are synthesized as inactive pro-enzymes or pro-caspases. In apoptosis, the pro-caspases are processed by proteolytic cleavage to form active enzymes. For example, caspase-3 exists in cells as an inactive 32 kDa proenzyme, called pro-caspase-3. Pro-caspase-3 is cleaved into active 17 and 12 kDa subunits by upstream proteases to become active caspase-3. Caspases-2, -8, -9 and -10 are classified as signaling or "upstream" in the apoptosis pathway because long prodomains allow association with cell surface receptors such as FAS (CD95), TNFR-1 (CD120a), DR-3 or CARD domains. This observation suggests a proteolytic cascade as a mechanism for signaling. A proteolytic cascade exists that would activate the terminal event required for apoptosis in a way similar to that of the coagulation cascade seen with the closely related family of serine proteases. For example, caspase-4 activates pro-caspase-1; caspase-9 activates pro-caspase-3; and caspase-3 cleaves pro-caspase-6 and pro-caspase-7. Caspases play a critical role in the execution phase of apoptosis. Important targets of caspases include cytoplasmic and nuclear proteins such as keratin 18, poly ADP ribose polymerase (PARP) and lamins. Overexpression of caspase-3 induces apoptosis. Through the use of synthetic peptides, caspases have been divided into three groups based on the four amino acids amino-terminal to their cleavage site. Caspases-1, -4 and -5 prefer substrates containing the sequence WEXD (where X is variable). Caspases-2, -3 and -7 prefer the sequence DEXD. Caspases 6, 8 and 9 are the least demanding but have demonstrated a preference for cleaving of substrates containing either LEXD or VEXD. Because these sequences correspond to known cleavage sites of caspase targets, systems to study caspase cleavage activity have been developed. The measurement of caspase enzyme activity with fluorometric and colorimetric peptide substrates and the detection of caspase cleavage using antibodies to caspases allows the study of the apoptosis processes or screening of therapeutic agents which promote or prevent apoptosis. A typical assay would involve the cleavage of a fluorescent substrate peptide to quantitate activity. The substrate, DEVD-AFC, is composed of the fluorophore, AFC (7-amino-4-trifluoromethyl coumarin), and a synthetic tetrapeptide, DEVD (Asp-Glu-Val-Asp), which is the upstream amino acid sequence of the Caspase-3 cleavage site in PARP. DEVD-AFC emits blue light ($\lambda_{max} = 400 \text{ nm}$). Upon cleavage of the substrate by Caspase-3 or

related caspases, with the excitation wavelength set to 400 nm, free AFC emits a yellow-green fluorescence ($\lambda_{\text{max}} = 505 \text{ nm}$) which can be quantified using a spectrofluorometer or a fluorescence microtiter plate reader. Comparison of the fluorescence of AFC from apoptotic samples with an uninduced control allows
5 determination of the increase in caspase-3 activity. See Jaeschke, et al. J. Immunol., 160:3480-3486 (1998); Talanian, et al., J. Biol. Chem., 272:9677-9682 (1997).

Yet another example of an apoptosis assay is the TUNEL assay. Briefly, cell death by apoptosis is characterized by DNA fragmentation in 200-250 and/or 30-50 kilobases. Further internucleosomal DNA fragmentation in 180-200 base pairs may
10 also occur. Such characteristics have been used to distinguish apoptotic cells from normal or necrotic cells. To detect apoptotic cells, whatever the pattern of DNA fragmentation, the TUNEL (Terminal deoxynucleotidyl transferase (TdT) mediated dUTP Nick End Labeling) method is commonly utilized. One of the most easily measured features of apoptotic cells is the break-up of the genomic DNA by cellular
15 nucleases. These DNA fragments can be extracted from apoptotic cells and result in the appearance of "DNA laddering" when the DNA is analyzed by agarose gel electrophoresis. The DNA of non-apoptotic cells, which remains largely intact, does not display this "laddering" on agarose gels during electrophoresis. The large number of DNA fragments appearing in apoptotic cells results in a multitude of 3'-hydroxyl
20 ends in the DNA. This property can be used to identify apoptotic cells by labeling the 3'-hydroxyl ends with brominated deoxyuridine triphosphate nucleotides (Br-dUTP). The enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes a template independent addition of deoxyribonucleoside triphosphates to the 3'-hydroxyl ends of double- or single-stranded DNA with either blunt, recessed or overhanging ends. See
25 Li and Darzynkiewicz, Cell Prolif., 28:572-579 (1995).

In another apoptosis assay, the cell death ELISA detects the same endpoint as the TUNEL assay, DNA fragmentation. However, in the cell death ELISA assay, the histone complexed DNA fragments are measured directly by antibodies in an ELISA assay. See Piro, et al., Metabolism, 51:1340-7 (2002); Facchiano et al., Exp. Cell
30 Res., 271:118-29 (2001); Horigome et al., Immunopharmacology, 37:87-94 (1997).

The invention further provides a method of decreasing cell proliferation, comprising the application to said cell of an agent that down-modulates the activity of SEQ ID NO: 1 or SEQ ID NO:3. The agent can be a ribozyme, an antisense

molecule, an antagonizing antibody, an siRNA or a small organic molecule, as discussed above.

An example of a cell proliferation assay is the AlamarBlueTM assay, which is described for instance in Example 1. Other examples include the Natural Red, methylene blue and tetrazolium/formazan assays. See also biosource.com; Alley et al., Cancer Res., 48:589-601 (1988); Elliot and Auersperg, Biotech. Histochem., 68:29-35 (1993); and Scudiero et al., Cancer Res., 48:4827-33 (1988). Another example of a cell proliferation assay is based on the cleavage of the terazolium salt WST-1. biochem.roche.com/pack-insert/1644807a.pdf (Roche, Version 3, May, 1999).

Another example of an assay that measures a difference in cell proliferation (or tumorigenicity) is a soft agar assay, as described in Example 1. See also Bergo et al., Mol. and Cell. Biol., 22:171-181 (2002); Zeng et al., Cancer Res., 62:3538:3543 (2002).

The invention further provides a method of identifying a molecule that inhibits cancer cells by applying the molecule to cells, where the molecule down-modulates the RNA correlate of SEQ ID NOS:1 or 3. The method further provides measuring the level of down-modulation of the compound, where an increase in level of down-modulation indicates that said molecule inhibits cancer cells.

Measurement of down-modulation can be made by a reporter assay using a reporter gene operably linked to a nucleic acid encoding GPR56 or DNER. Reporter genes can express proteins such as β -lactamase, luciferase, green fluorescent protein, β -galactosidase, secreted alkaline phosphatase, human growth hormone and chlororamphenicol acetyltransferase.

The invention also provides a method of identifying a compound that inhibits cancer cells by applying the compound to cells, where it down-modulates SEQ ID NOS:2 or 4. The method further provides measuring the level of down-modulation of the compound, where an increase in level of down-modulation indicates that said molecule inhibits cancer cells. Down-modulation can be measured, for example, by an immunoassay using an antibody specific to said compound. Such an immunoassay can be, for example, an immunofluorescence, immunochemistry or immunoprecipitation assay.

As used herein, the term “ribozyme” refers to catalytic RNA molecules that bind to the target nucleic acid molecules (GPR56 or DNER) and cleave them, thereby impairing their ability to function in cancer growth and development. They may be “hairpin” ribozymes, “hammerhead” ribozymes or any other type of ribozyme known in the art.

As used herein, the term “nucleic acid” or “nucleic acid molecule” refers to deoxyribonucleotides or ribonucleotides, oligomers and polymers thereof, in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid. For example, as disclosed herein, such analogues include those with substitutions, such as methoxy, at the 2-position of the sugar moiety. Unless otherwise indicated by the context, the term is used interchangeably with gene, cDNA and mRNA encoded by a gene.

As used herein, the phrase “a nucleotide sequence encoding” refers to a nucleic acid which contains sequence information, for example, for a ribozyme, mRNA, siRNA, and the like, or for the primary amino acid sequence of a specific protein or peptide. In reference to a ribozyme, unless otherwise indicated, the explicitly specified encoding nucleotide sequence also implicitly covers sequences that do not materially effect the specificity of the ribozyme for its target nucleic acid. In reference to a protein or peptide, unless otherwise indicated, the explicitly specified encoding nucleotide sequence also implicitly encompasses variations in the base sequence encoding the same amino acid sequence (*e.g.*, degenerate codon substitutions). The invention also contemplates proteins or peptides with conservative amino acid substitutions. The identity of amino acids that may be conservatively substituted is well known to those of skill in the art. Degenerate codons of the native sequence or sequences may be chosen to conform with codon preference in a specific host cell.

As used herein, the term “RNA correlate” of a given DNA sequence means that sequence with “U” substituted for “T,” with the entire sequence in ribonucleic acid form. The present invention encompasses the RNA correlates of SEQ ID NOS:1, and 3.

As applied to any of the methods of the subject invention, the terms “sequence similarity”, “sequence identity”, or “percent identity,” in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or

subsequences that are, when optimally aligned with appropriate nucleotide insertions or deletions, the same or have a specified percentage of amino acid residues or nucleotides that are the same (i.e., 50% identity, 65%, 70%, 75%, 80%, preferably 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or higher identity to an amino acid sequences such as SEQ ID NOS:2 and 4 (or domains thereof), or a nucleotide sequence such as SEQ ID NOS:1 and 3 (or RNA correlates thereof), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. This definition also refers to the complement of a test sequence. Preferably, the identity exists over a region that is at least about 25 amino acids or nucleotides in length, or more preferably over a region that is 50-100 amino acids or nucleotides in length. These relationships hold, notwithstanding evolutionary origin (Reeck *et al.*, *Cell*, **50**:667 (1987)). When the sequence identity of a pair of polynucleotides or polypeptides is greater or equal to 65%, the sequences are said to be "substantially identical."

Alternatively, substantial identity will exist when a nucleic acid will hybridize under selective hybridization conditions, to a strand or its complement. Typically, selective hybridization will occur when there is at least about 55% homology over a stretch of at least about 14 nucleotides, more typically at least about 65%, preferably at least about 75%, and more preferably at least about 90%. See, Kanehisa, *Nuc. Acids Res.*, **12**:203-213 (1984), which is incorporated herein by reference. The length of homology comparison, as described, may be over longer stretches, and in certain embodiments will be over a stretch of at least about 17 nucleotides, generally at least about 20 nucleotides, ordinarily at least about 24 nucleotides, usually at least about 28 nucleotides, typically at least about 32 nucleotides, more typically at least about 40 nucleotides, preferably at least about 50 nucleotides, and more preferably at least about 75 to 100 or more nucleotides.

Amino acid sequence homology, or sequence identity, is determined by optimizing residue matches, if necessary, by introducing gaps as required. This changes when considering conservative substitutions as matches. Conservative substitutions typically include substitutions within the following groups: [glycine, alanine]; [valine, isoleucine, leucine]; [aspartic acid, glutamic acid]; [asparagine, glutamine]; [serine, threonine]; [lysine, arginine]; and [phenylalanine, tyrosine]. Homologous amino acid sequences are intended to include natural allelic and

interspecies variations in each respective receptor sequence. Typical homologous proteins or peptides will have from 25-100% homology (if gaps can be introduced), to 50-100% homology (if conservative substitutions are included). Homology measures will be at least about 50%, generally at least 56%, more generally at least 62%, often at least 67%, more often at least 72%, typically at least 77%, more typically at least 82%, usually at least 86%, more usually at least 90%, preferably at least 93%, and more preferably at least 96%, and in particularly preferred embodiments, at least 98% or more.

In relation to proteins, the term "homology" in all its grammatical forms refers to the relationship between proteins that possess a "common evolutionary origin," including proteins from superfamilies (e.g., the immunoglobulin superfamily) and homologous proteins from different species (e.g., myosin light chain, *etc.*) (Reeck *et al.*, *Cell*, **50**:667 (1987)). The present invention naturally contemplates homologues of the GPR56 protein disclosed herein, and polynucleotides encoding the same, as falling within the scope of the invention.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* **2**:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* **48**:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* **85**:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and

TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (*see, e.g., Current Protocols in Molecular Biology* (Ausubel *et al.*, eds. 1995 supplement)).

One example of a useful algorithm is PILEUP. PILEUP creates a multiple
5 sequence alignment from a group of related sequences using progressive, pairwise
alignments to show relationship and percent sequence identity. It also plots a tree or
dendrogram showing the clustering relationships used to create the alignment.
PILEUP uses a simplification of the progressive alignment method of Feng &
Doolittle, *J. Mol. Evol.* **35**:351-360 (1987). The method used is similar to the method
10 described by Higgins & Sharp, *CABIOS* **5**:151-153 (1989). The program can align up
to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids.
The multiple alignment procedure begins with the pairwise alignment of the two most
similar sequences, producing a cluster of two aligned sequences. This cluster is then
aligned to the next most related sequence or cluster of aligned sequences. Two
15 clusters of sequences are aligned by a simple extension of the pairwise alignment of
two individual sequences. The final alignment is achieved by a series of progressive,
pairwise alignments. The program is run by designating specific sequences and their
amino acid or nucleotide coordinates for regions of sequence comparison and by
designating the program parameters. Using PILEUP, a reference sequence is
20 compared to other test sequences to determine the percent sequence identity
relationship using the following parameters: default gap weight (3.00), default gap
length weight (0.10), and weighted end gaps. PILEUP can be obtained from the GCG
sequence analysis software package, e.g., version 7.0 (Devereaux *et al.*, *Nuc. Acids*
Res. **12**:387-395 (1984).

25 Another example of algorithm that is suitable for determining percent
sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms,
which are described in Altschul *et al.*, *Nuc. Acids Res.* **25**:3389-3402 (1977) and
Altschul *et al.*, *J. Mol. Biol.* **215**:403-410 (1990), respectively. Software for
performing BLAST analyses is publicly available through the National Center for
30 Biotechnology Information (ncbi.nlm.nih.gov). This algorithm involves first
identifying high scoring sequence pairs (HSPs) by identifying short words of length
W in the query sequence, which either match or satisfy some positive-valued
threshold score T when aligned with a word of the same length in a database
sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*,

supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

An indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the antibodies raised against the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their

complements hybridize to each other under stringent conditions, as described below. Yet another indication that two nucleic acid sequences are substantially identical is that the same primers can be used to amplify the sequence.

It should be understood that each method of the invention described herein encompasses: a) all compounds having about 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identity with SEQ ID NOs:1 or 3 and RNA correlates of SEQ ID NOS:1 or 3; b) all compounds with 50, 60, 70, 80, 90 or more amino acids and having about 92%, 95%, 96%, 97%, 98% or 99% identity with SEQ ID NOS:2 or 4; and c) all compounds with 100, 150, 200, 250, 300 or more nucleotides and having about 92%, 95%, 96%, 97%, 98% or 99% identity with SEQ ID NOS:1 or 3 or the RNA correlates thereof

The term "moderately stringent conditions," as used herein, means hybridization conditions that permit a nucleic acid molecule to bind to a second nucleic acid molecule that has substantial identity to the sequence of the first. Moderately stringent conditions are those equivalent to hybridization of filter-bound nucleic acid in 50% formamide, 5 X Denhart's solution, 5 X SSPE, 0.2% SDS at 42°C, followed by washing in 0.2 X SSPE, 0.2% SDS at 50°C. "Highly stringent conditions" are those equivalent to hybridization of filter-bound nucleic acid in 50% formamide, 5 X Denhart's solution, 5 X SSPE, 0.2% SDS at 42°C, followed by washing in 0.2 X SSPE, 0.2% SDS at 65°C. Other suitable moderately stringent and highly stringent conditions are known in the art and described, for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1992), and Ansel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore MD (1998).

In general, a nucleic acid molecule that hybridizes to a second one under moderately stringent conditions will have greater than about 60% identity, preferably greater than about 70% identity and, more preferably, greater than about 80% identity over the length of the two sequences being compared. A nucleic acid molecule that hybridizes to a second one under highly stringent conditions will have greater than about 90% identity, preferably greater than about 92% identity and, more preferably, greater than about 95%, 96%, 97%, 98% or 99% identity over the length of the two sequences being compared.

As used herein, the term "isolated" when used in conjunction with a nucleic acid or protein, denotes that the nucleic acid or protein has been isolated with respect

to the many other cellular components with which it is normally associated in the natural state. For example, an "isolated" gene of interest may be one that has been separated from open reading frames which flank the gene and encode a gene product other than that of the specific gene of interest. Such genes may be obtained by a number of methods including, for example, laboratory synthesis, restriction enzyme digestion or PCR. Likewise, an "isolated" protein may be substantially purified from a natural source or may be synthesized in the laboratory. A "substantially purified" nucleic acid or protein gives rise to essentially one band in an electrophoretic gel, and is at least 85% pure, more preferably at least 95% pure, and most preferably at least 99% pure.

The term "intrabody" refers to a class of neutralizing molecules with applications in gene therapy (vonMehren M, Weiner L M. (1996) *Current Opinion in Oncology*. 8:493-498, Marasco Wash. (1997) *Gene Therapy*. 4:11-15, Rondon I J, Marasco Wash. (1997) *Annual Review of Microbiology*. 51:257-283). Intrabodies are engineered antibodies that can be expressed within a cell and target an intracellular molecule of interest. Using this technique, intracellular signals and enzyme activities can be inhibited, or their transport to cellular compartments prevented. Marasco, W. A., et al., Proc. Natl. Acad. Sci. USA 90:7889-7893 (1993). Thus, intrabodies provide yet another approach to down regulating GPR56 expression and activity.

The intrabody method is analogous to the inactivation of proteins by deletion or mutation, but is directed at the level of gene product rather than at the gene itself. Using the intrabody strategy even molecules involved in essential cellular pathways can be targeted, modified or blocked. Antibody genes for intracellular expression can be derived either from murine or human monoclonal antibodies or from phage display libraries. For intracellular expression small recombinant antibody fragments, containing the antigen recognizing and binding regions, can be used. Intrabodies can be directed to different intracellular compartments by targeting sequences attached to the antibody fragments. The construction and use of intrabodies is discussed, for example, in U.S. Pat. No.: 6,004,940.

As used herein, the term "expression vector" includes a recombinant expression cassette that has a nucleotide sequence that can be transcribed into RNA in a cell. The cell can further translate transcribed mRNA into protein. An expression vector can be a plasmid, virus, or nucleic acid fragment. Typically, the recombinant expression cassette portion of an expression vector includes the encoding nucleotide

sequence to be transcribed (e.g. a ribozyme or siRNA), operably linked to a promoter, or other regulatory sequence by a functional linkage in *cis*. In accordance with the present invention, an expression vector comprising a nucleotide sequence encoding ribozymes of the invention can be used to transduce cells suitable as hosts for the vector. Both procaryotic cells including bacterial cells such as *E. coli* and eukaryotic cells including mammalian cells may be used for this purpose.

As used herein, the term "promoter" includes nucleic acid sequences near the start site of transcription (such as a polymerase binding site) and, optionally, distal enhancer or repressor elements (which may be located several thousand base pairs from the start site of transcription) that direct transcription of the nucleotide sequence in a cell. The term includes both a "constitutive" promoter such as a pol III promoter, which is active under most environmental conditions and stages of development or cell differentiation, and an "inducible" promoter, which initiates transcription in response to an extracellular stimulus, such as a particular temperature shift or exposure to a specific chemical. Promoters and other regulatory elements (e.g., an origin of replication), and/or chromosome integration elements such as retroviral long terminal repeats ("LTRs"), or adeno associated viral (AAV) inverted terminal repeats ("ITRs"), may be incorporated into an expression vector encoding ribozymes of the present invention as described in WO 00/05415 to Barber *et al*.

As used herein, the term "expresses" denotes that a given nucleic acid comprising an open reading frame is transcribed to produce an RNA molecule. It also denotes that a given nucleic acid is transcribed and translated to produce a polypeptide. Although the term may be used to refer to the transcription of a ribozyme, a ribozyme typically is not translated into a protein since it functions as an active (catalytic) nucleic acid.

As used herein, the term "gene product" refers either to the RNA produced by transcription of a given nucleic acid or to the polypeptide produced by translation of a given nucleic acid.

As used herein, the term "transduce" denotes the introduction of an exogenous nucleic acid molecule (e.g., by means of an expression vector) inside the membrane of a cell. Exogenous DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. The exogenous DNA may be maintained on an episomal element, such as a plasmid. In eukaryotic cells, a stably transduced cell is generally one in which the exogenous DNA has become integrated

into the chromosome so that it is inherited by daughter cells through chromosome replication, or one which includes stably maintained extrachromosomal plasmids. 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 exogenous DNA.

The term "transfection," as used herein, means the genetic modification of a cell by uptake of an exogenous nucleic acid molecule (*e.g.*, by means of an expression vector).

As used herein, the term "ribozyme gene vector library" denotes a collection of ribozyme-encoding genes, typically within expression cassettes, in a collection of viral or other vectors. The vectors may be naked or contained within a capsid. Propagation of a ribozyme gene vector library can be performed as described in WO 00/05415 to Barber *et al.* The ribozyme-encoding genes of a ribozyme gene vector library, after transduction and transcription in appropriate cells, produce a collection of ribozymes.

As used herein, "small interfering RNAs" (siRNA) are short double-stranded RNA fragments that elicit a process known as "RNA interference" (RNAi), a form of sequence-specific gene silencing. Zamore, Phillip *et al.*, Cell 101:25-33(2000); Elbashir, Sayda M., *et al.*, Nature 411:494-497 (2001). SiRNAs are assembled into a multi-component complex known as the RNA-induced silencing complex (RISC). The siRNAs guide RISC to homologous mRNAs, thus targeting them for destruction. Hammond *et al.*, Nature Genetics Reviews 2:110-119(2000). RNAi has been observed in a variety of organisms including plants, insects and mammals, and cultured cells derived from these organisms.

An "siRNA" is a double-stranded RNA that is preferably between 16 and 25, more preferably 17 and 23 and most preferably between 18 and 21 base pairs long, each strand of which has a 3' overhang of 2 or more nucleotides. Functionally, the characteristic distinguishing an siRNA over other forms of dsRNA is that the siRNA comprises a sequence capable of specifically inhibiting genetic expression of a gene or closely related family of genes by a process termed RNA interference.

siRNAs for use in the present invention can be produced from nucleic acid sequences encoding GPR56 or DNER (SEQ ID NOS:1 or 3). For example, short complementary DNA strands are first prepared that represent portions of both the "sense" and "antisense" strands of the GPR56 coding region. This is typically

accomplished using solid phase nucleic acid synthesis techniques, as known in the art. The short duplex DNA thus formed is ligated into a suitable vector that is then used to transfect a suitable cell line. Other methods for producing siRNA molecules are known in the art. (See, e.g., Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001). For a review of RNAi and siRNA expression, see Hammond, Scott M. *et al.*, *Nature Genetics Reviews*, **2**:110-119; Fire, Andrew (1999) *TIG*, **15**(9):358-363; Bass, Brenda L. (2000) *Cell*, **101**:235-238. An siRNA of the invention can be constructed using, for example, a Lentiviral vector for stable expression.

siRNA molecules can be transfected into a cell line, for example HeLa, by using an agent such as OligofectamineTM, as described in Example 1. See also Invitrogen Corp., Transfecting siRNA into HeLa Cells Using OligofectamineTM, Doc. Rev. 102902 (Carlsbad, CA); Elbashir, et al., *Nature*, 411:494-498 (2001); and Harborth et al., *Science*, 114:4557-4565 (2001).

The targeting of antisense oligonucleotides to mRNA is another mechanism of decreasing protein synthesis, and, consequently, represents a powerful and targeted approach to diminishing expression of GPR56 or DNER. For example, the synthesis of polygalacturonase and the muscarine type 2 acetylcholine receptor are inhibited by antisense oligonucleotides directed to their respective mRNA sequences (U.S. Pat. Nos. 5,739,119 and 5,759,829, each specifically incorporated herein by reference in its entirety). Further, examples of antisense inhibition have been demonstrated with the nuclear protein cyclin, the multiple drug resistance gene (MDG1), ICAM-1, E-selectin, STK-1, striatal GABA.sub.A receptor and human EGF (Jaskulski *et al.*, 1988; Vasanthakumar and Ahmed, 1989; Peris *et al.*, 1998; U.S. Pat. Nos. 5,801,154; 5,789,573; 5,718,709 and 5,610,288, each specifically incorporated herein by reference in its entirety). Antisense constructs have also been described that inhibit and can be used to treat a variety of abnormal cellular proliferations, e.g. cancer (U.S. Pat. Nos. 5,747,470; 5,591,317 and 5,783,683, each specifically incorporated herein by reference in its entirety).

The invention provides therefore oligonucleotide sequences that comprise all, or a portion of, any sequence that is capable of specifically binding to a polynucleotide sequence described herein, or a complement thereof. In one embodiment, the antisense oligonucleotides comprise DNA or derivatives thereof. In another embodiment, the oligonucleotides comprise RNA or derivatives thereof. In a

third embodiment, the oligonucleotides are modified DNAs comprising a phosphorothioated modified backbone. In a fourth embodiment, the oligonucleotide sequences comprise peptide nucleic acids or derivatives thereof. In each case, preferred compositions comprise a sequence region that is complementary, and more preferably substantially-complementary, and even more preferably, completely complementary to one or more portions of SEQ ID NOS:1 or 3.

Selection of antisense compositions specific for a given gene sequence is based upon analysis of the chosen target sequence and determination of secondary structure, binding energy, and relative stability. Antisense compositions are selected based upon their relative inability to form dimers, hairpins, or other secondary structures that would reduce or prohibit specific binding to the target mRNA in a host cell.

“Non-native promoter” refers to any promoter element operably linked to a coding sequence by recombinant methods. Non-native promoters include mutagenized native reporters, when mutagenesis alters the rate or control of transcriptional events.

“Operably linked” refers to a linkage of polynucleotide elements in a functional relationship. With regard to the present invention, the term “operably linked” refers to a functional linkage between a nucleic acid expression control sequence (such as a promoter, or an array of transcription factor binding sites) and a second nucleic acid sequence, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence. Thus, a nucleic acid is “operably linked” when it is placed into a functional relationship with another nucleic acid sequence.

The invention also encompasses vectors in which a GPR56 or DNER nucleic acid is cloned into a vector in reverse orientation, but operably linked to a regulatory sequence that permits transcription of antisense RNA. Thus, an antisense transcript can be produced to all, or to a portion, of the nucleic acid sequences described herein, including both coding and non-coding regions. Expression of this antisense RNA is subject to each of the parameters described above in relation to expression of the sense RNA (regulatory sequences, constitutive or inducible expression, tissue-specific expression).

Antisense nucleic acids may be obtained from libraries encoding GPR56 or DNER, or synthesized synthetically. Transfection of suitable host cells with such a protein is performed in a manner analogous to that described for siRNAs above.

“Recombinant expression cassette” refers to a DNA sequence capable of directing expression of a nucleic acid in cells. A “DNA expression cassette” comprises a promoter, operably linked to a nucleic acid of interest, which is further operably linked to a termination region.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

Polypeptides often contain amino acids other than the 20 amino acids commonly referred to as the 20 naturally-occurring amino acids. Further, many amino acids, including the terminal amino acids, may be modified by natural processes, such as processing and other post-translational modifications, or by chemical modification techniques well known in the art. Common modifications that occur naturally in polypeptides are described in basic texts, detailed monographs, and the research literature, and they are well known to those of skill in the art.

Accordingly, the polypeptides also encompass derivatives or analogs in which a substituted amino acid residue is not one encoded by the genetic code, in which a substituent group is included, in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence for purification of the mature polypeptide or a pro-protein sequence.

Known modifications include, but are not limited to, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a

heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent crosslinks, formation of cystine, formation of pyroglutamate, formylation, gamma carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination.

Such modifications are well-known to those of skill in the art and have been described in great detail in the scientific literature. Several particularly common modifications, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, for instance, are described in most basic texts, such as *Proteins--Structure and Molecular Properties*, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York (1993). Many detailed reviews are available on this subject, such as by Wold, F., *Posttranslational Covalent Modification of Proteins*, B. C. Johnson, Ed., Academic Press, New York 1-12 (1983); Seifter *et al.* (*Meth. Enzymol.* **182**: 626-646 (1990)) and Rattan *et al.* (*Ann. N.Y. Acad. Sci.* **663**:48-62 (1992)).

As is also well known, polypeptides are not always entirely linear. For instance, polypeptides may be branched as a result of ubiquitination, and they may be circular, with or without branching, generally as a result of post-translation events, including natural processing event and events brought about by human manipulation which do not occur naturally. Circular, branched and branched circular polypeptides may be synthesized by non-translational natural processes and by synthetic methods.

Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. Blockage of the amino or carboxyl group in a polypeptide, or both, by a covalent modification, is common in naturally-occurring and synthetic polypeptides. For instance, the amino terminal residue of polypeptides made in *E. coli*, prior to proteolytic processing, almost invariably will be N-formylmethionine.

The modifications can be a function of how the protein is made. For recombinant polypeptides, for example, the modifications will be determined by the host cell posttranslational modification capacity and the modification signals in the polypeptide amino acid sequence. Accordingly, when glycosylation is desired, a

polypeptide should be expressed in a glycosylating host, generally a eukaryotic cell. Insect cells often carry out the same posttranslational glycosylations as mammalian cells and, for this reason, insect cell expression systems have been developed to efficiently express mammalian proteins having native patterns of glycosylation.

5 Similar considerations apply to other modifications.

The same type of modification may be present in the same or varying degree at several sites in a given polypeptide. Also, a given polypeptide may contain more than one type of modification.

Candidate protein-based compounds for binding or down-modulating the
10 proteins of the invention or one of its domains include, for example, 1) peptides such as soluble peptides, including fusion peptides and members of random peptide libraries (see, e.g., Lam *et al.*, Nature **354**:82-84 (1991); Houghten *et al.*, Nature **354**:84-86 (1991)) and combinatorial chemistry-derived molecular libraries made of L- and/or D-configuration amino acids; 2) phosphopeptides (e.g., members of random
15 and partially degenerate, directed phosphopeptide libraries, see, e.g., Songyang *et al.*, Cell **72**:767-778 (1993)); 3) antibodies (e.g., polyclonal, monoclonal, humanized, anti-idiotypic, chimeric, and single chain antibodies, including intrabodies, as well as Fab, F(ab').sub.2, Fab expression library fragments, and epitope-binding fragments of antibodies); and 4) small organic and inorganic molecules (e.g., molecules obtained
20 from combinatorial and natural product libraries).

Soluble full-length receptors, or fragments of the same, that compete for ligand binding are also considered candidate reagents. Other candidate compounds include mutant receptors or appropriate fragments containing mutations that affect receptor function and thus compete for ligand. Accordingly, a fragment that competes
25 for ligand, for example with a higher affinity, or a fragment that binds ligand but does not allow release, is encompassed by the invention. The receptor polynucleotides are also useful for constructing host cells expressing a part, or all, of the receptor polynucleotides and polypeptides.

The receptor polynucleotides are also useful for constructing transgenic
30 animals expressing all, or a part, of the receptor polynucleotides and polypeptides. These animals are useful as model systems for the treatment of cancer and can be used to test compounds for their effect, through the receptor gene or gene product, on the development or progression of the disease.

As used herein, the term "knockdown" or "down-modulation" means a decrease in the rate or level of mRNA production and/or protein production. For example, siRNA constructs of the present invention were shown to down-modulate the mRNA of GPR56 and DNER (SEQ ID NOS:1 and 3), as discussed above and in the examples below.

The present invention also provides a method of inhibiting melanoma, lung, breast, prostate, cervical, glioma (or other brain or CNS cancers), colon or ovarian cancer cells or their growth. This method includes applying an agent of the invention to the cancer cells. Such an agent can be, for example, a ribozyme of the present invention, an antisense molecule, an antibody or an antagonizing antibody or an siRNA. This method can comprise transducing the infected cell with an expression vector encoding the agent. Alternatively, the agent can be introduced into a cell directly, i.e., without using a vector.

The present invention also discloses methods for treating malignancies. Hereinafter, the term "treatment" includes both the prevention of the genesis of the malignancy, as well as the substantial reduction or elimination of malignant cells or symptoms associated with the development and metastasis of malignancies.

Tumors or malignancies for which the molecules or compounds of the present invention are useful include all metastatic tumors. Examples of tumors for which such a treatment would be effective include, but are not limited to, breast cancers such as infiltrating duct carcinoma of the breast or other metastatic breast cancers, lung cancers such as small cell lung carcinoma, bone cancers, pancreatic cancers, bladder cancers such as bladder carcinoma, rhabdomyosarcoma, angiosarcoma.

adenocarcinoma of the colon, prostate or pancreas, or other metastatic prostate or colon cancers, squamous cell carcinoma of the cervix, ovarian cancer, malignant fibrous histiocytoma, skin cancers such as malignant melanoma, lymphomas, leukemia, leiomyosarcoma, astrocytoma, glioma and hepatocellular carcinoma. Such treatment may optionally and preferably be performed by systemic administration of the therapeutic agent according to the present invention. A preferred route of administration is oral. Alternative routes of administration include, but are not limited to, intranasal, intraocular, sub-cutaneous and parenteral administration. Such treatment may be performed topically, for example for skin malignancies, including but not limited to, metastatic melanoma. Other routes of administration and suitable pharmaceutical formulations thereof are also possible as previously described.

The compounds of the present invention can be used to treat a variety of conditions, including, but not limited to, those listed in U.S. Pat. No. 5,861,382.

The method of the invention can be accomplished by the agent binding to a target, for instance SEQ ID NOS: 1 or 3, or portions or domains thereof, as discussed above. The method of the invention can also be accomplished by the agent down-modulating SEQ ID NOS:1 or 3, or portions or domains thereof, as discussed above.

In accordance with another embodiment of the present invention, the step of down-modulating the level of the target protein in the cell can be accomplished by introducing into the cell system an antisense compound or molecule.

Combinatorial peptide libraries can be screened to identify antagonists of a protein of the invention or one of their domains, which can increase the inhibition of cancer cells. Combinatorial peptide libraries can be constructed from genomic or cDNA libraries, or by using non-cellular synthetic methods. Techniques for solid phase synthesis are described by Barany and Merrifield, *Solid-Phase Peptide Synthesis*, pp. 3-284 in *The Peptides: Analysis, Synthesis, Biology*. Vol. 2: Special Methods in Peptide Synthesis, Part A.; Merrifield, *et al.*, *J. Am. Chem. Soc.* **85**: 2149-2156 (1963), and Stewart *et al.*, *Solid Phase Peptide Synthesis*, 2nd ed., Pierce Chem. Co., Rockford, Ill. (1984). Proteins may be synthesized by condensation of the amino and carboxy termini of shorter fragments. Methods of forming peptide bonds by activation of a carboxy terminal end (e.g., by the use of the coupling reagent N,N'-dicyclohexylcarbodiimide) are known to those of skill.

The proteins useful in this invention may be purified to substantial purity by standard techniques well known in the art, including detergent solubilization, selective precipitation with such substances as ammonium sulfate, column chromatography, immunopurification methods, and others. See, for instance, R. Scopes, *Protein Purification: Principles and Practice*, Springer-Verlag: New York (1982); Deutscher, *Guide to Protein Purification*, Academic Press (1990). For example, antibodies may be raised to the proteins as described herein. Purification from *E. coli* can be achieved following procedures described in U.S. Pat. No. 4,511,503.

Peptide and protein reagents can optionally be labeled, as described below, or may be used in the screening assays of the present invention to ascertain their ability to modulate GPR56 expression or activity.

Portions of SEQ ID NOS:2 or 4 can be useful in competition binding assays in methods designed to discover compounds that interact with the receptor. Thus, a

compound is exposed to a receptor polypeptide under conditions that allow the compound to bind or to otherwise interact with the polypeptide. Soluble receptor polypeptide is also added to the mixture. If the test compound interacts with the soluble receptor polypeptide, it decreases the amount of complex formed or activity from the receptor target. This type of assay is particularly useful in cases in which compounds are sought that interact with specific regions of the receptor. Thus, the soluble polypeptide that competes with the target receptor region is designed to contain peptide sequences corresponding to the region of interest.

The compounds tested as modulators of proteins or protein domains of the invention or RNA correlates of the invention can be any small chemical compound, or a biological entity, such as a protein, sugar, nucleic acid or lipid. Screening combinatorial libraries of small organic molecules offers an approach to identifying useful therapeutic compounds or precursors targeted to proteins or protein domains of the invention or RNA correlates of the invention. Typically, test compounds will be small chemical molecules and peptides. Essentially any chemical compound can be used as a potential modulator or ligand in the assays of the invention, although most often compounds can be dissolved in aqueous or organic (especially DMSO-based) solutions are used. The assays are designed to screen large chemical libraries by automating the assay steps and providing compounds from any convenient source to assays, which are typically run in parallel (e.g., in microtiter formats on microtiter plates in robotic assays). It will be appreciated that there are many suppliers of chemical compounds, including Sigma (St. Louis, MO), Aldrich (St. Louis, MO), Sigma-Aldrich (St. Louis, MO), Fluka Chemika-Biochemica Analytika (Buchs Switzerland) and the like.

In one embodiment, high throughput screening methods are utilized involving a combinatorial chemical or peptide library containing a large number of potential therapeutic compounds (potential modulator or ligand compounds). Such “combinatorial chemical libraries” or “ligand libraries” are screened in one or more assays, as described herein, to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity. The compounds thus identified can serve as conventional “lead compounds” or can themselves be used as potential or actual therapeutics.

A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis, by

combining a number of chemical “building blocks” such as reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining a set of chemical building blocks (amino acids) in every possible way for a given compound length (i.e., the number of amino acids in a polypeptide compound).

5 Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks. Preparation and screening of combinatorial chemical libraries is well known to those of skill in the art.

To perform cell-free drug screening assays, it is desirable to immobilize either the receptor protein, or fragment, or its target molecule to facilitate separation of
10 complexes from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay.

In one embodiment, the invention provides soluble assays using molecules such as a ligand binding domain, an extracellular domain, a transmembrane domain (e.g., one comprising seven transmembrane regions and cytosolic loops), the
15 transmembrane domain and a cytoplasmic domain, an active site, a subunit association region, etc.; a domain that is covalently linked to a heterologous protein to create a chimeric molecule; the full-length protein of the invention; or a cell or tissue expressing such protein, either naturally occurring or recombinant. In another embodiment, the invention provides solid phase based *in vitro* assays in a high
20 throughput format, where the domain, chimeric molecule, protein of the invention, or cell or tissue expressing such protein is attached to a solid phase substrate.

In the high throughput assays of the invention, it is possible to screen up to several thousand different modulators or ligands in a single day. In particular, each well of a microtiter plate can be used to run a separate assay against a selected
25 potential modulator, or, if concentration or incubation time effects are to be observed, every 5-10 wells can test a single modulator. Thus, a single standard microtiter plate can assay about 100 (e.g., 96) modulators. If 1536 well plates are used, then a single plate can easily assay from about 100 to about 1500 different compounds. It is possible to assay several different plates per day; assay screens for up to about 6,000-
30 20,000 different compounds is possible using the integrated systems of the invention. More recently, microfluidic approaches to reagent manipulation have been developed, e.g., by Caliper Technologies (Palo Alto, CA).

The molecule of interest can be bound to the solid state component, directly or indirectly, via covalent or non covalent linkage e.g., via a tag. The tag can be any of a

variety of components. In general, a molecule which binds the tag (a tag binder) is fixed to a solid support, and the tagged molecule of interest is attached to the solid support by interaction of the tag and the tag binder.

5 A number of tags and tag binders can be used, based upon known molecular interactions well described in the literature. For example, where a tag has a natural binder, for example, biotin, protein A, or protein G, it can be used in conjunction with appropriate tag binders (avidin, streptavidin, neutravidin, the Fc region of an immunoglobulin, *etc.*) Antibodies to molecules with natural binders such as biotin are also widely available as are appropriate tag binders; *see*, SIGMA Immunochemicals
10 1998 catalogue SIGMA, St. Louis MO).

Similarly, any haptenic or antigenic compound can be used in combination with an appropriate antibody to form a tag/tag binder pair. Thousands of specific antibodies are commercially available and many additional antibodies are described in the literature. For example, in one common configuration, the tag is a first antibody
15 and the tag binder is a second antibody that recognizes the first antibody. In addition to antibody-antigen interactions, receptor-ligand interactions are also appropriate as tag and tag-binder pairs. For example, agonists and antagonists of cell membrane receptors (e.g., cell receptor-ligand interactions such as transferrin, c-kit, viral receptor ligands, cytokine receptors, chemokine receptors, interleukin receptors,
20 immunoglobulin receptors and antibodies, the cadherein family, the integrin family, the selectin family, and the like; *see, e.g.*, Pigott & Power, *The Adhesion Molecule Facts Book I* (1993). Similarly, toxins and venoms, viral epitopes, hormones (e.g., opiates, steroids, *etc.*), intracellular receptors (e.g. which mediate the effects of various small ligands, including steroids, thyroid hormone, retinoids and vitamin D;
25 peptides), drugs, lectins, sugars, nucleic acids (both linear and cyclic polymer configurations), oligosaccharides, proteins, phospholipids and antibodies can all interact with various cell receptors.

Synthetic polymers, such as polyurethanes, polyesters, polycarbonates, polyureas, polyamides, polyethyleneimines, polyarylene sulfides, polysiloxanes,
30 polyimides, and polyacetates can also form an appropriate tag or tag binder. Many other tag/tag binder pairs are also useful in assay systems described herein, as would be apparent to one of skill upon review of this disclosure.

Common linkers such as peptides, polyethers, and the like can also serve as tags, and include polypeptide sequences, such as poly gly sequences of between about

5 and 200 amino acids. Such flexible linkers are known to persons of skill in the art. For example, poly(ethelyne glycol) linkers are available from Shearwater Polymers, Inc. Huntsville, Alabama. These linkers optionally have amide linkages, sulfhydryl linkages, or heterofunctional linkages.

5 Tag binders are fixed to solid substrates using any of a variety of methods currently available. Solid substrates are commonly derivatized or functionalized by exposing all or a portion of the substrate to a chemical reagent which fixes a chemical group to the surface which is reactive with a portion of the tag binder. For example, groups which are suitable for attachment to a longer chain portion would include
10 amines, hydroxyl, thiol, and carboxyl groups. Aminoalkylsilanes and hydroxyalkylsilanes can be used to functionalize a variety of surfaces, such as glass surfaces. The construction of such solid phase biopolymer arrays is well described in the literature. See, e.g., Merrifield, *J. Am. Chem. Soc.* **85**:2149-2154 (1963) (describing solid phase synthesis of, e.g., peptides); Geysen *et al.*, *J. Immun. Meth.* **102**:259-274 (1987) (describing synthesis of solid phase components on pins); Frank & Doring, *Tetrahedron* **44**:60316040 (1988) (describing synthesis of various peptide sequences on cellulose disks); Fodor *et al.*, *Science*, **251**:767-777 (1991); Sheldon *et al.*, *Clinical Chemistry* **39**(4):718-719 (1993); and Kozal *et al.*, *Nature Medicine* **2**(7):753759 (1996) (all describing arrays of biopolymers fixed to solid substrates).
15
20 Non-chemical approaches for fixing tag binders to substrates include other common methods, such as heat, cross-linking by UV radiation, and the like.

Another approach uses recombinant bacteriophage to produce large libraries. Using the "phage method" (Scott and Smith, *Science* **249**:386-390, 1990; Cwirla, *et al.*, *Proc. Natl. Acad. Sci.*, **87**:6378-6382, 1990; Devlin *et al.*, *Science*, **49**:404-406, 1990), very large libraries can be constructed (10^6 - 10^8 chemical entities). A second approach uses primarily chemical methods, of which the Geysen method (Geysen *et al.*, *Molecular Immunology* **23**:709-715, 1986; Geysen *et al.*, *J. Immunologic Method* **102**:259-274, 1987; and the method of Fodor *et al.* (*Science* **251**:767-773, 1991) are examples. Furka *et al.* (14th International Congress of Biochemistry, Volume #5, Abstract FR:013, 1988; Furka, *Int. J. Peptide Protein Res.* **37**:487-493, 1991),
25
30 Houghton (U.S. Pat. No. 4,631,211, issued December 1986) and Rutter *et al.* (U.S. Pat. No. 5,010,175, issued Apr. 23, 1991) describe methods to produce a mixture of peptides that can be tested as agonists or antagonists.

In another aspect, synthetic libraries (Needels *et al.*, *Proc. Natl. Acad. Sci. USA* **90**:10700-4, 1993; Ohlmeyer *et al.*, *Proc. Natl. Acad. Sci. USA* **90**:10922-10926, 1993; Lam *et al.*, International Patent Publication No. WO 92/00252; Kocis *et al.*, International Patent Publication No. WO 9428028) and the like can be used to screen
5 for a protein or domain according to the present invention.

The screening can be performed with recombinant cells that express the proteins or domains thereof of the invention, or alternatively, using purified proteins, e.g., produced recombinantly, as described above. For example, the ability of labeled, soluble or solubilized GPR56 that includes the ligand-binding portion of the molecule,
10 to bind ligand can be used.

Yet another assay for compounds that modulate the activity of GPR56 or DNER involves computer assisted drug design, in which a computer system is used to generate a three-dimensional structure of a protein of the invention based on the structural information encoded by the amino acid sequence. The input amino acid
15 sequence interacts directly and actively with a pre-established algorithm in a computer program to yield secondary, tertiary, and quaternary structural models of the protein. The models of the protein structure are then examined to identify regions of the structure that have the ability to bind, e.g., ligands. These regions are then used to identify ligands that bind to the protein.

20 The three-dimensional structural model of the protein is generated by entering protein amino acid sequences of at least 10 amino acid residues or corresponding nucleic acid sequences encoding GPR56 or DNER into the computer system. Contiguous portions of SEQ ID NOS:1 or 3 and conservatively modified versions thereof, can be used for this purpose. The amino acid sequence represents the primary
25 sequence or subsequence of the protein, which encodes the structural information of the protein. At least 10 residues of the amino acid sequence (or a nucleotide sequence encoding 10 amino acids) are entered into the computer system from computer keyboards, computer readable substrates that include, but are not limited to, electronic storage media (e.g., magnetic diskettes, tapes, cartridges, and chips), optical media
30 (e.g., CD ROM), information distributed by internet sites, and by RAM. The three-dimensional structural model of the protein is then generated by the interaction of the amino acid sequence and the computer system, using software known to those of skill in the art.

The amino acid sequence represents a primary structure that encodes the information necessary to form the secondary, tertiary and quaternary structure of the protein of interest. The software looks at certain parameters encoded by the primary sequence to generate the structural model. These parameters are referred to as
5 “energy terms,” and primarily include electrostatic potentials, hydrophobic potentials, solvent accessible surfaces, and hydrogen bonding. Secondary energy terms include van der Waals potentials. Biological molecules form the structures that minimize the energy terms in a cumulative fashion. The computer program therefore uses these terms encoded by the primary structure or amino acid sequence to create the
10 secondary structural model.

The tertiary structure of the protein encoded by the secondary structure is then formed on the basis of the energy terms of the secondary structure. The user at this point can enter additional variables such as whether the protein is membrane bound or soluble, its location in the body, and its cellular location, e.g., cytoplasmic, surface, or
15 nuclear. These variables along with the energy terms of the secondary structure are used to form the model of the tertiary structure. In modeling the tertiary structure, the computer program matches hydrophobic faces of secondary structure with like, and hydrophilic faces of secondary structure with like.

Once the structure has been generated, potential ligand binding regions are
20 identified by the computer system. Three-dimensional structures for potential ligands are generated by entering amino acid or nucleotide sequences or chemical formulas of compounds, as described above. The three-dimensional structure of the potential ligand is then compared to that of GPR56 or DNER to identify ligands that bind to such protein. Binding affinity between the protein and ligands is determined using
25 energy terms to determine which ligands have an enhanced probability of binding to the protein.

The activity of the proteins of the invention can be assessed using a variety of *in vitro* and *in vivo* assays that determine functional, physical and chemical effects, e.g., measuring ligand binding (e.g., by radioactive ligand binding), second
30 messengers (e.g., cAMP, cGMP, IP₃, DAG, or Ca²⁺), ion flux, phosphorylation levels, transcription levels, neurotransmitter levels, and the like. Furthermore, such assays can be used to test for inhibitors and activators of proteins or domains of the invention. Modulators can also be genetically altered versions of GPR56 or DNER. Such modulators can be useful in the treatment and diagnosis of cancer.

The polypeptide of the assay will be selected from SEQ ID NOS:2 or 4, a portion of 10, 20, 30, 40, 50 or more contiguous amino acids thereof, or conservatively modified variants thereof. Alternatively, the protein of the assay will be derived from a eukaryote and include an amino acid subsequence where the
5 homology will be at least 60%, preferably at least 75%, more preferably at least 90% and most preferably between 95% and 100% that of SEQ ID NOS:2 or 4. Optionally, the polypeptide of the assays will comprise a domain of SEQ ID NOS:2 or 4. Either SEQ ID NOS: 2 or 4 or a domain thereof can be covalently linked to a heterologous protein to create a chimeric protein used in the assays described herein.

10 Modulators of the activity of GPR56 or DNER are tested using polypeptides as described above, either recombinant or naturally occurring. The protein can be isolated, expressed in a cell, expressed in a membrane derived from a cell, expressed in tissue or in an animal, either recombinant or naturally occurring.

Changes in ion flux may be assessed by determining changes in polarization
15 (i.e., electrical potential) of the cell or membrane expressing a protein of the invention. One means to determine changes in cellular polarization is by measuring changes in current (thereby measuring changes in polarization) with voltage-clamp and patch-clamp techniques, e.g., the "cell-attached" mode, the "inside-out" mode, and the "whole cell" mode (*see, e.g., Ackerman et al., New Engl. J. Med.* **336**:1575-
20 1595 (1997)). Whole cell currents are conveniently determined using the standard methodology (*see, e.g., Hamil et al., Pflugers. Archiv.* **391**:85 (1981)). Other known assays include: radiolabeled ion flux assays and fluorescence assays using voltage-sensitive dyes (*see, e.g., Vestergaard-Bogind et al., J. Membrane Biol.* **88**:67-75 (1988); Gonzales & Tsien, *Chem. Biol.* **4**:269-277 (1997); Daniel *et al., J. Pharmacol. Meth.* **25**:185-193 (1991); Holevinsky *et al., J. Membrane Biology* **137**:59-70 (1994)).
25 Generally, the compounds to be tested are present in the range from 1 pM to 100 mM.

The practice of using a reporter gene to analyze nucleotide sequences that regulate transcription of a gene-of-interest is well documented. The demonstrated utility of a reporter gene is in its ability to define domains of transcriptional regulatory
30 elements of a gene-of-interest. Reporter genes express proteins that serve as detectable labels indicating when the control elements regulating reporter gene expression are up or down-regulated in response to outside stimuli.

By way of example, two types of reporter gene assay are discussed below. The first is a scorable reporter gene, whose expression can be quantified, giving a

proportional indication of the level of expression supported by the genetic construct comprising the reporter gene. The second example is a selectable reporter gene. When expressed, the selectable reporter gene allows the host cell harboring the reporter gene to survive under restrictive conditions that would otherwise kill (or
5 retard the growth of) the host cell.

Scorable reporter genes are typically used when the relative activity of a genetic construct is sought, whereas selectable reporters are used when confirmation of the presence of the reporter expression construct within the cell is desired.

Firefly luciferase expression systems have become widely used for
10 quantitative analysis of transcriptional modulation in living cells (see, e.g., Wood, K.V. (1998) *Promega Notes* 65:14). In particular, recombinant cells comprising this reporter construct enable libraries of small molecules to be rapidly screened for those affecting specific aspects of cellular physiology, such as receptor function or intracellular signal transduction. deWet *et al.* (1987) *Mol. Cell. Biol.* 7:725; Wood,
15 K.V. (1991) In: *Bioluminescence and Chemiluminescence: Current Status*, eds. P. Stanley and L. Kricka, John Wiley and Sons, Chichester, 11.

The luciferase assay could be used to screen any of the potential reagents listed above. For example, by creating a fusion protein comprising the luciferase and GPR56 coding sequences, siRNAs, antisense sequences and ribozymes targeted
20 against the GPR56 gene can be screened, as any reagent acting on the GPR56 transcript will necessarily disrupt expression of the luciferase enzyme encoded in the same transcript.

Modulators will manifest themselves by altering the amount of light emitted by the luciferase-catalyzed hydrolysis of ATP, with up-modulators increasing the
25 amount of light emitted (they induce increased luciferase production) and down-modulators decreasing the amount of light emitted (by inhibiting luciferase production) in proportion to the degree of expressional modulation (at least within the linear range limits of the assay). Luciferase assay kits and other reporter gene constructs suitable for use in the present invention are well known in the art and
30 commercially available, e.g., Invitrogen and Promega. See, e.g., *Steady-Glo™ Luciferase Assay Reagent Technical Manual Luciferase Assay Reagent Technical Manual* #TM051, Promega Corporation.

A number of selectable marker systems can be used in the present invention, including but not limited to the herpes simplex virus thymidine kinase (Wigler, *et al.*,

1977, *Cell* **11**:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, *Proc. Natl. Acad. Sci. USA* **48**:2026), and adenine phosphoribosyltransferase (Lowy, *et al.*, 1980, *Cell* **22**:817) genes can be employed in *tk*⁻, *hgp^rt*⁻ or *ap^rt*⁻ cells, respectively. Also, antimetabolite resistance can be used as
5 the basis of selection for *dhfr*, which confers resistance to methotrexate (Wigler, *et al.*, 1980, *Natl. Acad. Sci. USA* **77**:3567; O'Hare, *et al.*, 1981, *Proc. Natl. Acad. Sci. USA* **78**:1527); *gpt*, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, *Proc. Natl. Acad. Sci. USA* **78**:2072); *neo*, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, *et al.*, 1981, *J. Mol. Biol.* **150**:1); and
10 *hygro*, which confers resistance to hygromycin (Santerre, *et al.*, 1984, *Gene* **30**:147) genes.

Typically, selectable markers are included in expression cassettes comprising the target gene or construct to be incorporated into the host cell. The selectable marker may be under the control of the same promoter as the target construct, e.g., as
15 part of a fusion protein or polycistronic transcript; or may be under the control of an independent promoter.

As suggested above, the purpose of the selectable marker is to confer selectable growth characteristics on cells that are able to express it. By including the selectable marker in the same nucleic acid comprising the target gene or construct, the
20 selectable marker will be included in any cell transformed with the target. Therefore, by selecting for the growth characteristics conferred by the selectable marker, cells transfected with the target can be selected.

Real-time PCR assays, as described in the examples herein, take advantage of those cycles of a normal PCR reaction where the DNA being amplified is increasing
25 at a logarithmic rate and hence proportional to the amount of DNA present. Several kits are commercially available for performing real-time PCR. One such kit is the TaqMan® assay.

The TaqMan® assay takes advantage of the 5' nuclease activity of Taq DNA polymerase to digest a DNA probe annealed specifically to the accumulating
30 amplification product. TaqMan® probes are labeled with a donor-acceptor dye pair that interacts via fluorescence energy transfer. Cleavage of the TaqMan® probe by the advancing polymerase during amplification dissociates the donor dye from the quenching acceptor dye, greatly increasing the donor fluorescence. All reagents necessary to detect two allelic variants can be assembled at the beginning of the

reaction and the results are monitored in real time. In an alternative homogeneous hybridization based procedure, molecular beacons are used for allele discriminations. Molecular beacons are hairpin-shaped oligonucleotide probes that report the presence of specific nucleic acid molecules in homogeneous solutions. When they bind to their targets they undergo a conformational reorganization that restores the fluorescence of an internally quenched fluorophore. (See, e.g., Heid, C.A., Stevens, J., Livak, K.J. and Williams, P.M. Real time quantitative PCR. *Genome Res.* 6:986-994 (1996); Gibson, U.E.M., Heid, C.A. and Williams, P.M. A novel method for real time quantitative RT-PCR. *Genome Res.* 6:995-1001 (1996)).

Northern blot methods allow RNA isolated from cells of interest to be separated using gel electrophoresis techniques. After separation, nucleic acids are transferred to membranes and hybridized with radio-labeled nucleotide probes. For analysis of expression maps, poly A (adenylyl) probed are used, which hybridize to mRNA species present on the blot.

The present invention includes both traditional and expression map Northern blotting. Expression of SEQ ID NOS:1 and 3 and other genes of interest can be tracked using probes specific for these genes. Expression mapping can be used to monitor alterations in gene expression in response to GPR56-specific or DNER-specific binding agents.

Methods of RNA isolation are taught in, for example, Ausubel, F. M. *et al.*, Current Protocols in Molecular Biology, Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Northern blot analysis is routine in the art and is taught in, for example, Ausubel, F. M. *et al.*, Current Protocols in Molecular Biology, Volume 1, pp. 4.2.1-4.2.9, John Wiley & Sons, Inc., 1996.

Through the use of high density oligonucleotide arrays, expression profiles for individual cells can be rapidly obtained and compared. High density arrays are particularly useful for monitoring expression control at the transcriptional, RNA processing and degradation level. The fabrication and application of high density arrays in gene expression monitoring have been disclosed in, for example, WO 97/10365, WO 92/10588, U.S. Pat. No. 6,040,138 incorporated herein for all purposes by reference. In some embodiments using high density arrays, high density oligonucleotide arrays are synthesized using methods such as the Very Large Scale Immobilized Polymer Synthesis (VLSIPS) disclosed in U.S. Pat. No. 5,445,934. Each oligonucleotide occupies a known location on a substrate. A nucleic acid target

sample is hybridized with a high density array of oligonucleotides and then the amount of target nucleic acids hybridized to each probe in the array is quantified. One preferred quantifying method is to use confocal microscope and fluorescent labels. The GeneChip.RTM. system (Affymetrix, Santa Clara, Calif.) is particularly
5 suitable for quantifying the hybridization; however, it will be apparent to those of skill in the art that any similar systems or other effectively equivalent detection methods can also be used.

High density arrays are suitable for quantifying a small variations in expression levels of a gene in the presence of a large population of heterogeneous
10 nucleic acids. Such high density arrays can be fabricated either by *de novo* synthesis on a substrate or by spotting or transporting nucleic acid sequences onto specific locations of substrate. Nucleic acids are purified and/or isolated from biological materials, such as a bacterial plasmid containing a cloned segment of sequence of interest. Suitable nucleic acids are also produced by amplification of templates. As a
15 nonlimiting illustration, polymerase chain reaction or *in vitro* transcription are suitable nucleic acid amplification methods.

Synthesized oligonucleotide arrays are particularly preferred for this invention. Oligonucleotide arrays have numerous advantages, as opposed to other methods, such as efficiency of production, reduced intra- and inter array variability,
20 increased information content and high signal-to-noise ratio.

An "antisense compound or molecule" refers to such compound or molecule that includes a polynucleotide that is complementary to a target sequence of choice and capable of specifically hybridizing with the target molecules. The term antisense includes a "ribozyme," which is a catalytic RNA molecule that cleaves a target RNA
25 through ribonuclease activity. Antisense nucleic acids hybridize to a target polynucleotide and interfere with the transcription, processing, translation or other activity of the target polynucleotide. An antisense nucleic acid can inhibit DNA replication or DNA transcription by, for example, interfering with the attachment of DNA or RNA polymerase to the promoter by binding to a transcriptional initiation
30 site or a template. It can interfere with processing of mRNA, poly(A) addition to mRNA or translation of mRNA by, for example, binding to regions of the RNA transcript such as the ribosome binding site. It can promote inhibitory mechanisms of the cells, such as promoting RNA degradation via RNase action. The inhibitory polynucleotide can bind to the major groove of the duplex DNA to form a triple

helical or "triplex" structure. Methods of inhibition using antisense polynucleotides therefore encompass a number of different approaches to altering expression of specific genes that operate by different mechanisms (see, e.g., Helene & Toulme, *Biochim. Biophys. Acta.*, **1049**:99-125 (1990)).

5 The antisense compounds that may be used in connection with this embodiment of the present invention preferably comprise between about 8 to about 30 nucleobases (*i.e.*, from about 8 to about 30 linked nucleosides), more preferably from about 12 to about 25 nucleobases, and may be linear or circular in configuration. They may include oligonucleotides containing modified backbones or non-natural
10 internucleoside linkages. Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and
15 aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Methods of preparing antisense compounds are well known in the art (see, for example, U.S. Patent No.
20 6,210,892).

 The present invention also provides a method of increasing inhibition of cancer cells, the method comprising introducing into the cell an effective amount of an expression vector comprising a sequence of nucleotides that encodes an siRNA or a ribozyme having a suitable substrate binding sequence. The expression vector is
25 preferably administered in combination with a suitable carrier. After the vector has been administered, the ribozyme or siRNA is expressed in the cell.

 This method can be applied to a subject with cancer. Administration of the vector into the subject can be by any suitable route including oral, sublingual intravenous, subcutaneous, transcutaneous, intramuscular, intracutaneous, and the
30 like. Any of a variety of non-toxic, pharmaceutically acceptable carriers can be used for formulation including, glucose, lactose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea, dextrans, and the like. The formulated material may take any of various forms

such as injectable solutions, sterile aqueous or non-aqueous solutions, suspensions or emulsions, tablets, capsules, and the like.

As used herein, the phrase “effective amount” refers to a dose of the deliverable sufficient to provide circulating concentrations high enough to impart a beneficial effect on the recipient, which is an increase of inhibition of cancer cells.

The specific therapeutically effective dose level for any particular subject and deliverable depends upon a variety of factors including the severity of the infection, the activity of the specific compound administered, the route of administration, the rate of clearance of the specific compound, the duration of treatment, the drugs used in combination or coincident with the specific compound, the age, body weight, sex, diet and general health of the patient, and like factors well known in the medical arts and sciences. Dosage levels typically range from about 0.001 up to 100 mg/kg/day; with levels in the range of about 0.05 up to 10 mg/kg/day.

The present invention also provides an antibody with binding specificity for a protein of the invention, such as SEQ ID NOS:2 or 4, or any molecule with 80%, 85%, 90% or 95% or more sequence identity with SEQ ID NOS:2 or 4, or any fragment of 10 or more contiguous amino acids of SEQ ID NOS:2 or 4. Examples of such antibodies include GD3 to GD8, as described in Example 1. The antibody can have a binding specificity for a protein or peptide (i.e., amino acid sequence) encoded by SEQ ID NOS:1 or 3, or any molecule with 80%, 85%, 90% or 95% or more sequence identity with SEQ ID NOS:1 or 3.

As used herein, the term “antibody” refers to a polypeptide comprising a framework region from an immunoglobulin gene or fragments thereof that specifically binds and recognizes an antigen. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

An exemplary immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one “light” (about 25 kDa) and one “heavy” chain (about 50-70 kDa). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable

light chain (V_L) and variable heavy chain (V_H) refer to these light and heavy chains respectively.

Antibodies can exist as intact immunoglobulins or as a number of well-characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce $F(ab)'_2$, a dimer of Fab which itself is a light chain joined to V_H - C_{H1} by a disulfide bond. The $F(ab)'_2$ may be reduced under mild conditions to break the disulfide linkage in the hinge region, thereby converting the $F(ab)'_2$ dimer into an Fab' monomer. The Fab' monomer is essentially Fab with part of the hinge region (see *Fundamental Immunology* (Paul ed., 3d ed. 1993)). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized *de novo* either chemically or by using recombinant DNA methodology.

Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies, or those synthesized *de novo* using recombinant DNA methodologies (e.g., single chain Fv) or those identified using phage display libraries (see, e.g., McCafferty *et al.*, *Nature* 348:552-554 (1990)).

Methods of producing polyclonal and monoclonal antibodies are known to those of skill in the art. See, e.g., Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; and Harlow and Lane (1989) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Press, NY; Stites *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA; Huse *et al.* (1989) *Science* 246:1275-1281; and Ward *et al.* (1989) *Nature* 341:544-546, and references cited therein. Techniques for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce antibodies to polypeptides of this invention.

Also, transgenic mice, or other organisms such as other mammals, may be used to express humanized antibodies. Alternatively, phage display technology can be used to identify antibodies and heteromeric Fab fragments that specifically bind to selected antigens (see, e.g., McCafferty *et al.*, *Nature* 348:552-554 (1990); Marks *et al.*, *Biotechnology* 10:779-783 (1992)).

Methods of producing polyclonal and monoclonal antibodies that react specifically with GPR56 of the invention are known to those of skill in the art (see, e.g., Coligan, *Current Protocols in Immunology* (1991); Harlow & Lane, *supra*;

Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed. 1986); and Kohler & Milstein, *Nature* **256**:495-497 (1975). Such techniques include antibody preparation by selection of antibodies from libraries of recombinant antibodies in phage or similar vectors, as well as preparation of polyclonal and monoclonal antibodies by
5 immunizing rabbits or mice (*see, e.g., Huse et al., Science* **246**:1275-1281 (1989); Ward *et al., Nature* **341**:544-546 (1989)).

A number of proteins comprising immunogens may be used to produce antibodies specifically reactive with SEQ ID NOS: 2 or 4, or portions thereof. For example, recombinant GPR56 or an antigenic fragment thereof can be isolated, as is
10 known in the art. Recombinant protein can be expressed in eukaryotic or prokaryotic cells as described above, and purified as generally described above. Recombinant protein is one embodiment of an immunogen for the production of monoclonal or polyclonal antibodies. Alternatively, a synthetic peptide derived from the sequences disclosed herein and conjugated to a carrier protein can be used as an immunogen.
15 Naturally occurring protein may also be used either in pure or impure form. The product is then injected into an animal capable of producing antibodies. Either monoclonal or polyclonal antibodies may be generated, for subsequent use in immunoassays to measure the protein.

Methods of production of polyclonal antibodies are known to those of skill in
20 the art. An inbred strain of mice (e.g., BALB/C mice) or rabbits is immunized with the protein using a standard adjuvant, such as Freund's adjuvant, and a standard immunization protocol. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the GPR56 protein. When appropriately high titers of antibody to the immunogen are
25 obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the protein can be done if desired (*see Harlow & Lane, supra*).

Monoclonal antibodies may be obtained by various techniques familiar to those skilled in the art. Briefly, spleen cells from an animal immunized with a desired
30 antigen are immortalized, commonly by fusion with a myeloma cell (*see Kohler & Milstein, Eur. J. Immunol.* **6**:511-519 (1976)). Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods well known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity

and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells may be enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host. Alternatively, one may isolate DNA sequences which encode a monoclonal antibody or a binding fragment thereof by screening a DNA
5 library from human B cells according to the general protocol outlined by Huse *et al.*, *Science* **246**:1275-1281 (1989).

Monoclonal antibodies and polyclonal sera are collected and titered against the immunogen protein in an immunoassay, for example, a solid phase immunoassay with the immunogen immobilized on a solid support. Typically, polyclonal antisera
10 with a titer of 10^4 or greater are selected and tested for their cross reactivity against other related proteins, using a competitive binding immunoassay. Specific polyclonal antisera and monoclonal antibodies will usually bind with a K_d of at least about 0.1 mM, more usually at least about 1 μ M, optionally at least about 0.1 μ M or better, and optionally 0.01 μ M or better.

Once specific antibodies are available, the proteins of the invention can be
15 detected by a variety of immunoassay methods. For a review of immunological and immunoassay procedures, see *Basic and Clinical Immunology* (Stites & Terr eds., 7th ed. 1991). Moreover, the immunoassays of the present invention can be performed in any of several configurations, which are reviewed extensively in *Enzyme*
20 *Immunoassay* (Maggio, ed., 1980); and Harlow & Lane, *supra*.

Detection can be facilitated by coupling (i.e., physically linking) the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes
25 include horseradish peroxidase, alkaline phosphatase, β -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a
30 luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S or ^3H .

The antibodies are also useful for inhibiting receptor function, for example, blocking ligand binding. These uses can also be applied in a therapeutic context in

which treatment involves inhibiting receptor function. An antibody can be used, for example, to block ligand binding. Antibodies can be prepared against specific fragments containing sites required for function or against intact receptor associated with a cell.

5 As used herein, the phrase "binding specificity," in relationship to an antibody that binds to a protein or peptide, refers to a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibody binds to a particular protein and does not bind significantly to other
10 proteins present in the sample.

The following examples are intended to illustrate but not limit the present invention.

Example 1

1. General.

15 GPR56, also named TM7XN1, belongs to the family of G-protein coupled receptors (GPCRs). GPCRs are cell surface receptors typically consisting of seven transmembrane regions along with a N-terminal extracellular domain and a C-terminal cytoplasmic tail. GPCRs are involved in a variety of signal transduction pathways. The interaction between the GPCR and the intracellular effectors is mediated by heterotrimeric GTP-binding proteins. Baldwin, Curr. Opin. Cell Biol., 6:180-190 (1994); Strader et al., Annu. Rev. Biochem., 63:101-132 (1994).
20

GPR56 belongs to the secretin-like receptor family. It has a large N-terminal
25 extracellular region (amino acids 26 – 402) and contains a novel cysteine box located just before the first transmembrane spanning domain.

RNA transcripts of GPR56 have a wide tissue distribution with highest levels found in the thyroid gland, brain and heart. Liu et al., Genomics, 55: 296-305 (1999). Expression of GPR56 was shown to be upregulated in tumors of the brain as
30 compared to normal brain tissue. Several other types of tumors stained positive for GPR56 expression, including adenocarcinoma, renal cell carcinoma and non-melanoma carcinoma. Patent Application US 2003/0175209 A1. Zendman et al. describes a possible correlation between GPR56 expression and the metastatic potential of cancer cell lines. Zendman et al., FEBS Letters, 446: 292-298 (1999).

2. Protein information

Expression construct: Part of the HE26 gene covering the signal sequence and the first extracellular domain (amino acids 1-398 of SEQ ID NO:2) was cloned into the mammalian expression vector pEE14.4. Six histidine codons were added to the 3' end, so that the expressed protein contains a C-terminal His-tag. Note that amino acid 306 is His, whereas amino acid according to GenBank entry is Gln; this is a known polymorphism.

The Signal sequence of the expressed protein will be cleaved while the protein is secreted. The cleavage occurs between residues 25 and 26. The mature protein (as it is present in the supernatant of transfected cells) should have a molecular weight of about 43 kDA.

3. Expression of GPR56 in cancer cells

The expression level of GPR56 mRNA was assessed in the following cell lines: A2058 (melanoma), M14 (melanoma), UACC62 (melanoma), UACC257 (melanoma), HeLa (cervical cancer), HF (nontransformed derivative of HeLa), HCT116 (colon cancer), DLD1 (colon cancer), PC3 (prostate cancer), A549 (lung cancer), OVCAR3 (ovarian cancer), OVCAR8 (ovarian cancer), A431 (skin cancer), A2780 (ovarian cancer), U87 (glioma), U138 (glioma), NCI-H522 (non-small cell lung cancer; "NSCL"), NCI-H460 (NSCL), MDA-MB231-luc (breast cancer), HCT116-luc (colon cancer), WI38 (normal lung fibroblast), IMR90 (normal lung fibroblast) and HUVEC (normal endothelial cell).

RNA samples from sub-confluent cultures were prepared by standard procedures and real-time PCR (RT-PCR) was performed on these samples. The results are shown in Figures 3A and 14 (relative mRNA levels depicted as arbitrary units; GPR56 mRNA level normalized to the internal ribosomal 18S RNA control level). These results show that GPR56 is very highly expressed in: a) melanoma cells (M14, UACC62 and UACC257); b) non-small cell lung cancer cells (NCI-H522); and ovarian cancer cells (OVCAR3). It is also expressed in varying degrees in many of the other cancer cell lines tested. These results coordinate well with a "virtual northern blot" carried out utilizing software available at falcon.roswellpark.org/DN/DN1.html which shows that GPR56 is highly up-regulated in skin cancer tissue (see Figure 3B).

4. siRNA knockdown of GPR56 in melanoma cancer cells

Two lentiviral vectors encoding GPR56-specific hairpin siRNAs were transiently or stably transduced into M14, A2058, HELA, PC3, OVCAR8, OVCAR3, AsPC1 and NCI-H460 cancer cell lines to assess down-regulation of the expression of GPR56 (SEQ ID NO:1) in these cell lines. Construction of lentiviral vectors and transduction of cells were performed according to Ke et al., BioTechniques 36:826-833 (2004). The target sequences for the two GPR56-specific siRNAs were:

GAAGGTGCACATGAACCTGCTGC (siRNA "B"; SEQ ID NO:5) and

GAAATGTGGCTCCAGTTGCTCTC (siRNA "C"; SEQ ID NO:6). The forward

PCR primers comprising the siRNA coding sequences used to construct the lentiviral vectors were:

TGCTGGATCCAAAAAGAAGGTGCACATGAACCTGCTGCTCTCTTGAAGC
AGCAGGTTTCATGTGCACCTTCAAACAAGGCTTTTCTCCAAGGG (SEQ ID
NO: 7) for siRNA "B" and

TGCTGGATCCAAAAAGAAATGTGGCTCCAGTTGCTCTCTCTTGAAGA
GAGCAACTGGAGCCACATTTCAAACAAGGCTTTTCTCCAAGGG (SEQ ID
NO: 8) for siRNA "C". The reverse primer for both siRNA constructs was
GAACTAGTGGATCCGACGCC (SEQ ID NO: 9).

5. Validation Assays

The transduced cells were trypsinized and plated into 96-well plates. A number of cell-based validation assays were then performed to assess the phenotype resulting from the introduction of these siRNAs into the cells.

a. Anchorage-dependent growth

Cell growth in liquid culture was quantified on day 1 and day 3 (or day 4) by AlamarBlue™ staining (BioSource International, Inc., Camarillo, CA). The results of stable transduction of A2058 cells are shown in Figure 4A; the results of transient and stable transduction of M14 cells are shown in Figures 4B and 4C, respectively. These results show that both GPR56-specific siRNAs caused a significant reduction in anchorage-dependent growth of both A2058 cells and M14 cells.

b. Anchorage-independent growth

Cell growth in soft-agar culture was quantified on day 1 and day 7 (or day 8) by AlamarBlue™ staining. The results of stable transduction of A2058 cells are shown in Figure 5A; the results of transient and stable transduction of M14 cells are

shown in Figures 5B and 5C, respectively. These results show that both siRNAs targeting GPR56 also caused a significant reduction in anchorage-independent growth of both A2058 cells and M14 cells.

In addition, HeLa, A2058, PC3, OVCAR8, OVCAR3, AsPC1, NCI-H460 and M14 cells were stably transduced with lentiviral hairpin siRNA vectors against GPR56 (B & C) and control were generated as described (Ke et al., *supra*). 1000 cells of each of these cells with CNTL, B and C siRNAs were plated into the 96-well soft agar culture, and soft agar growth was scored 7 days later using AlamarBlue staining. The experiments were performed twice with three biological replicates. Error bar indicates standard deviation. See Figures 15B, 16B, 17B and 18A, 18B, 19A, 19B and 19C. These results also show that both siRNAs targeting GPR56 caused a significant reduction in anchorage-independent growth of all of these cells, with perhaps a modest reduction for one of the siRNAs in AsPC1.

In addition, using HELA and A2058 lines, 10000 cells from each of these lines were also plated in regular soft agar culture in the 10 cm dishes and allowed to grow for 27 days before colony numbers quantified by Qcount (Claassen et al., *Preclinica*, 2: 435-92004). See Figures 15C and 16C. These results also show that both siRNAs targeting GPR56 caused a significant reduction in anchorage-independent growth in both of these cell lines.

c. Down-regulation of GPR56 mRNA

mRNA was extracted from A2058, M14, HeLa and PC3 cell lines and stably transduced with one of the GPR56-specific siRNAs or with control siRNA and subjected to real-time RT-PCR (Taqman®) analysis. As can be seen from Figures 6A, 6B, 15A, 16A and 17A, the GPR56-specific siRNAs caused reduction in the GPR56 mRNA levels in all cell lines. These findings are consistent with the biological phenotype observed in the previous two experiments (reduction in anchorage-dependent growth in liquid culture and reduction in anchorage-independent growth in soft-agar).

6. Over-expression of GPR56

Since GPR56 plays role in cell transformation as shown by gene inactivation in cancer cell lines, it would be very interesting to know whether over-expression of GPR56 would have any phenotype. An open reading frame (ORF) was cloned into a retroviral vector under control of CMV promoter. The vector was used to transduce

melanoma cell line A2058 and murine fibroblast cell line NIH3T3 cells. The over-expression was confirmed by both real-time RT-PCR and Western blotting compared to cells with vector alone.

Next, siRNAs against GPR56 and the control siRNA were introduced into A2058 cells containing either the vector or GPR56 cDNA. Stable cells were generated and cell growth was determined by AlamarBlue proliferation assay (Ke et al., *supra*). The cell growth in liquid culture was determined after four days' growth in liquid culture. While siRNA B and C (SEQ ID NOS: 7 and 8, respectively) reduced cell growth dramatically in cells with vector alone, over-expression of GPR56 cDNA was able to rescue the growth almost to the level of cells with the control siRNA (Figure 20A). This further confirms that the reduced cell growth phenotype rendered by GPR56 siRNAs is GPR56 specific.

The pro-survival function of GPR56 cDNA implicates its oncogenic function. Stable NIH3T3 cells over-expressing human GPR56 were then generated. A foci-formation assay, which measures the cell growth on monolayer, and is the classic oncogenic assay, was conducted. An equal number of NIH3T3 cells with either the control vector or GPR56 cDNA were seeded in T225 flask so that the culture was confluent the next day. Cells were then allowed to grow for another one to two weeks until the foci appeared. The number of foci were counted and shown (Figure 20B). Over-expression of GPR56 in murine NIH3T3 increased the foci formation dramatically, thus establishing GPR56 as an oncogene.

Indeed, expression of GPR56 in tumor tissues was analyzed and found to be over-expressed in several cancers, as shown below:

	Sample number (# normal/# cancer)*	Fold-over-expression	% over-expressed #
Breast	10/10	1.5	5/10
Colon	10/10	2.5	7/10
NSCL	10/10	2.6	7/10
Ovarian	5/5	3.4	4/5
Pancreas	5/5	5.6	5/5
Melanoma	3/3	3.4	3/3
Astrocytoma	10/2	2.2	2/2

Note: * number of normal and cancer patients analyzed; # number of cancer patients that express > 150% of the average of normal patients.

7. Regression of staged tumors in response to induced GPR56 silencing in a xenograft model.

Whether GPR56 silencing affects tumor growth *in vivo* was next tested, using a modified xenograft athymic (*nu/nu*) mouse model. In this model, siRNA against GPR56 was expressed from a tetO-based inducible lentiviral vector transduced into human cancer cells prior to xenografting (Ke et al., *supra*). This model makes it possible to measure regression of different staged tumors in response to GPR56 inactivation, thus mimicking a commonly used clinical endpoint for real human cancer therapeutics.

Prostate cancer cell line PC3 cells were transduced with the inducible siRNA vectors against control and GPR56, and the stably transduced PC3 cells were generated under non-induced condition. The stable cells were next tested for growth in media with (induced) or without (non-induced) doxycycline, an inducer of Tet-regulated expression. While minimal difference in growth was observed between the induced and uninduced conditions for cells with control siRNA, cells with inducible GPR56 siRNA showed greatly reduced growth under induced condition, which correlated with GPR56 mRNA down-regulation (Figure 21A). Figure 21B shows detailed accumulated cell growth measured by alamarBlue staining over the time, which points out detectable phenotype starting on day 4.

With the induced phenotype confirmed *in vitro*, the non-induced cells were transplanted into athymic nude mice subcutaneously. Induction of GPR56 silencing *in vivo* was achieved by providing drinking water containing 2mg/ml of Doxycycline. Two induction schedules were applied in the experiments: 1) continuously dosing starting on day 0 after implantation, allowing tiny tumors (early staged) to establish prior to induction of the silencing (see above); 2) continuous dosing starting on day 16 post-implantation, allowing tumors to form completely before dosing with Doxycycline (referred to as later staged tumor). The tumor response to GPR56 silencing was assessed by tumor volumes measured twice a week ($=1/2(\text{length} \times \text{width} \times \text{width})$). In the first induction schedule, tumors were found to grow until day 10, after which regression was observed for cells containing inducible siRNA for GPR56. By day 17, no tumors could be detected and all 10 animals remained tumor-

free until day 37 (Figures 22A and 22B). Thus, GPR56 is essential for the growth of early staged tumors.

In order to evaluate the response for late staged tumors (the second induction schedule), tumors were allowed to grow to measurable sizes ($\sim 200 \text{ mm}^3$ in volume) under non-induced conditions until day 16. The animals with tumor sizes larger than 150 mm^3 (18 out of original 20 mice) for CNTL and 100 mm^3 (14 out of original 20 mice) for GPR56 were chosen and regrouped into two groups: 9 each for CNTL and 7 each for GPR56; so that the average tumor sizes are similar between the groups. One group was treated with doxycycline (DOX) starting on day 16, while the other group was not, to test the robustness of the tumor response to GPR56 silencing. Upon induction, the tumors silenced for GPR56 ceased to grow and tumor regression was observed at a slow but convincing rate for 80% (6/7) of the animals (Figure 22D) until day 28, after which some tumors started to re-grow. In contrast, tumors transduced with control vectors showed continued growth for all the animals (Figure 22C). This observed 100% (10/10) tumor regression response at early stage and 80% (6/7) tumor regression response for late stage tumors shows that GPR56 is a potent target for cancer therapy.

Two issues are worth noting. First, DOX itself also has some inhibitory effect on tumor growth, although it never caused regression (Figures 22A and 22C), consistent with previous reports (Saikali and Singh, *Anticancer Drugs*, 14:773-8 (2003)). It was then determined that a much lower dose would be sufficient for RNAi induction in the system of the subject invention, and should be able to minimize this non-specific effect in future experiments by reduction of DOX. Second, as noted previously, the regulated lentiviral siRNA vector always demonstrated slight gene silencing without induction ("leakiness"). Slower growth of GPR56 siRNA vector transduced tumors under non-induced condition was also seen. Successful application of this model to other targets will depend on the threshold of the target mRNA for phenotype induction.

8. Pathway analysis of GPR56 mediated transformation of A2058 cells.

GPR56 plays an important role in cell transformation. The down-stream genes that are regulated by GPR56 was next investigated, specifically the biological pathways via which GPR56 mediates cell transformation by comparing expression profiles of cells with or without a specific genetic event (in this case, GPR56

silencing). Comparison was made of gene expression profiles of A2058 cells with GPR56 siRNA vector or with a control vector, using Affymetrix GeneChip® analysis. Six hundred genes with down-regulated expression at the mRNA levels were identified in GPR56 silenced cells and over 200 genes were up regulated. Among the down-regulated genes are oncogenes FYN and RAS family proteins, consistent with the oncogenic nature of GPR56 (Figure 23). As a confirmation, Real-time RT-PCR was used to determine the mRNA expression for FYN and GPR56 in the cells expressing GPR56 siRNAs, and a correlation between the GPR56 and FYN down-regulation was observed (Figure 24A). Therefore, a reporter assay utilizing FYN promoter could be utilized for screening GPR56 antagonizing molecules.

The significantly altered genes were then examined against the known biological pathways to identify the potential pathways that may be involved in GPR56 gene expression and function. Bioinformatics tools can be used to accelerate the pathway identification process. Ingenuity provides a knowledge-based analysis that can be used to query pathway information based on a gene or gene list and the public data base. Using this analysis, the 600 down-regulated genes fit into a number of possible functional pathways, including the integrin mediated adhesion pathway (Figure 23). A partial list of the down-regulated genes involved in the integrin pathway is also listed in Table 1.

Table 1 Genes down-regulated by GPR56 silencing.

<u>Gene Symbol</u>	<u>Gene Title</u>
ITGA6	integrin, alpha 6
ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
ITGA4	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)
ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta
CYR61	cysteine-rich, angiogenic inducer, 61
CANX	Calnexin
DDR1	discoidin domain receptor family, member 1 /// discoidin domain receptor family, member 1
ITGB5	integrin, beta 5
LAMB1	laminin, beta 1 /// laminin, beta 1
LAMC1	laminin, gamma 1 (formerly LAMB2) /// laminin, gamma 1 (formerly LAMB2)
NID	nidogen (enactin)
PCDH7	BH-protocadherin (brain-heart)
PPP2R1B	protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), beta isoform
RAB13	RAB13, member RAS oncogene family /// RAB13, member RAS oncogene family

9. GPR56 silencing reduces cell adhesion

The above results suggest that GPR56 silencing impacts on integrin-mediated cell adhesion. Whether GPR56 gene inactivation affects cell adhesion was therefore examined. Fibronectin is a major type of ECM, and commonly used to assay cell adhesion (Hynes *Proc Natl Acad Sci USA*, 96:2588-90 (1999)). Comparison was made between A2058 cells with and without GPR56 silencing for cell adhesion by plating cells onto fibronectin-coated tissue culture plates. The cells that were able to adhere were then quantitated by alamarBlue staining after one hour. The results showed that GPR56 silenced cells exhibited significant reduction in cell adhesion (Figure 24B), confirming the involvement of GPR56 in a cell adhesion pathway.

10. Elevated anoikis

Detachment of cells from a substratum often leads to anoikis, (adhesion triggered apoptosis) which is one of the key factors contributing to a cell's incapability of anchorage-independent growth. The specific role of anoikis in the anchorage independent growth of A2058 cells was thereof re investigated. The cells were plated in either standard tissue culture medium (attached) or in methylcellulose semi-liquid media (detached) and incubated for 16 hours followed by a TUNEL-based ELISA assay to quantitate DNA fragmentation, an indication of apoptosis (anoikis). The results showed that significantly elevated levels of anoikis were observed for GPR56 silenced A2058 cells (Figure 24C), confirming that the reduction in soft-agar cloning efficiency at least in part results from an increase in anoikis.

11. Apoptosis assays

The reduction in cancer cell growth by the knockdown of GPR56 evidenced by the assays described above could result either from an increase in cell death (apoptosis) or a reduction in cell proliferation. To ascertain which of these two processes predominated, an apoptosis-specific assay was conducted that measures by ELISA the amount of DNA fragmentation of cells that have undergone apoptosis. M14, HCT116, and HeLa cells were seeded in 12-well plates at approximately 30% confluency and transduced with the siRNA constructs. 3 days post-transduction, the cells were harvested and assayed by ELISA in accordance with the manufacturer's instructions (Cell Death Detection ELISA; Roche Molecular Biochemicals). As can

be seen from results shown in Figure 7, knockdown by siRNA of GPR56 mRNA resulted in a significant increase in DNA fragmentation in the three cancer cell lines as compared to a control siRNA.

Moreover, further testing showed that knockdown of GPR56 results in an increase in cell death (apoptosis) selectively in cancer cells compared to normal (non-cancerous) cells. The same procedures described above were carried out in three non-cancerous cell lines (HUVEC, IMR90 and WI-38) and no DNA fragmentation was detected after transfection with GPR56-specific siRNA (see Figure 7). This indicates that agents targeting GPR56 are preferred candidates for therapeutic treatment because they can selectively induce apoptosis of the cancerous cells.

12. Surface expression of GPR56 and its cleavage at the putative GPS site

To examine the protein expression of GPR56, several rabbit polyclonal antibodies were raised against either peptides (GD3-6) or purified His-GPR56 extracellular domain (ECD, GD7-8) (Table 2).

Table 2

Protein	Antigen	Animal	Antiserum
GPR56	Extracellular domain (aa 1 – 399 of SEQ ID NO:2)	Rabbit	GD7 GD8
	Peptide: CSQRNQTHRSSLHYK (aa 35-49 of SEQ ID NO:2)	Rabbit	GD3 GD4
	Mixture of 3 peptides: RGHREDFRFDSARLPISC (aa 26-34, 677-684 of SEQ ID NO:2) HRSSLHYKPTDLRIC (aa 42-56 of SEQ ID NO:2) WKLQPTAGLQDLHIHC (aa 238-252 of SEQ ID NO:2)	Rabbit	GD5 GD6

The antibodies were tested using FACS (surface expression) and Western blot in melanoma cell line A2058, and the data with GD7 is shown. In FACS analysis, while the pre-immune didn't stain cells positively, first bleed gave surface staining, and the signal is higher in A2058 over-expressing GPR56 cDNA (Figure 25A), lower in A2058 expressing GPR56 siRNAs (Figure 25B). Similarly, the protein expression levels determined by Western blot using GPR56 antibodies also correlate with mRNA levels (Figure 26).

It is interesting to note that the size of GPR56 protein detected by Western blot is similar to that of the purified GPR56 ECD domain, which could be due to a

proteolytic event at the G-protein proteolytic site (GPS) site adjacent to the trans-membrane domain. The protein is also glycosylated, as treatment of the protein with PNGaseF decreases the molecular weight (Figure 26A). The surface expression of GPR56 is further confirmed by immuno-histochemistry (IHC) analysis of A2058 (Figure 27A) or A2058 over-expressing GPR56 (Figure 27B).

13. Treatment with GPR56 antibodies

After confirming the specificity of the antibodies with GPR56 antigen, and the surface staining of the GPR56 protein, the next determination was whether the antibody binding with GPR56 would block its function and have any effects on cell growth. Different dilutions (1:25, 1:50 or 1:100) of antibodies or their corresponding pre-immunes were incubated with melanoma cell line A2058 for two to five days, and cell growth was determined using AlamarBlue staining. Several antibodies (GD3, 5, 7) reduced the cell growth compared to their corresponding pre-immunes (Figure 28A, B, C), while the control antibody didn't give any growth phenotype (Figure 28D). In addition, the growth phenotype is dependent on the amount of the antibodies added, as the lower dilution of the antibody (1:25) gave more phenotype than higher dilutions. These results show that GPR56 antibodies, through binding with GPR56, blocks GPR56 function and thus interfere with cell growth.

Next, the effect of antibodies on cell lines that over-expresses GPR56 exogenously was tested to determine whether GPR56 over-expression would sensitize cells to antibody treatment. For these experiments, the cell pairs for human melanoma cell line A2058 and murine fibroblast cell line NIH3T3 were used. GD7 antibody was used since it gave the best phenotype as described above. For NIH3T3 cells, while GD7 has minimal effect on cell growth compared to pre-immune, it significantly reduced the cell growth of NIH3T3 cell over-expressing human GPR56 (Figures 29A and 29B). The sensitivity of A2058 cells was also greatly enhanced by over-expressing GPR56 in A2058 cell lines (Figures 29C and 29D). Thus, exogenous over-expressed GPR56 sensitizes cell growth to the antibody treatment. This is very significant, since many tumor tissues express much higher GPR56 than their normal counterparts, thus a differential killing of the tumor samples will be expected.

To further confirm that the antibody effect on cell growth was through recognizing GPR56 antigen, a neutralization assay was conducted. The antibody was incubated with different amounts of the antigen (purified GPR56 ECD) or control

protein (non-specific) for 30 minutes before they were added to the cells, and cell growth was measured four days later using AlamarBlue staining. If the GPR56 antigen neutralized the antibody, then no effect would be observed for cell growth, while the control antigen wouldn't have any neutralization effects. The data demonstrated that while both amounts of GPR56 antigen neutralized the growth inhibitory effect of the antibody (Figure 30A), the non-specific control antigen failed to neutralize the effect (Figure 30B). These results further confirmed that antibody effect on the cell growth is through recognizing the GPR56 antigen.

We have also determined the growth inhibitory effect of GD7 in other cell lines and significantly reduced growth was observed, as shown in Table 3:

Table 3. Cell lines tested for GD7 growth inhibitory effects

Cancer indication	Cell lines	Growth phenotype	Apoptosis
NSCL	A549	+++	
	H460	+++	
Colon	HCT116	+++	
Prostate	PC3	++	
Ovarian	OVCAR8	+++	
Pancreatic	AsPC1	++	
Melanoma	A2058	+++	+++
	UACC62	+++	

+++ >70% growth inhibition; ++ 40-60% growth inhibition.

It was next determined whether the growth inhibitory effect was due to apoptosis induction. For this experiment, both GD7 pre-immune and 2nd bleed were incubated with cells at different dilutions (1:25, 1:50, 1:100, and 1:200). Cells were harvested after overnight incubation, and levels of apoptosis were determined by a TUNNEL-based Elisa assay to determine DNA fragmentation, a hallmark of apoptosis. As shown in Figure 31A, the 2nd bleed induced pronounced apoptosis induction in melanoma line A2058 cells (~10 fold). In contrast, only minimal effect (<2 fold) was observed in renal primary epithelial cells HRE (Figure 31B) and blood PBMC cells (Figure 31C). This is consistent with the differential apoptosis induction observed in tumor cells in the siRNA experiment.

To show that the antibody effect is through binding to GPR56 to down-regulate GPR56 protein level, to facilitate the detection of GPR56 protein expression, the A2058/GPR56 cell line is used since it over-expressed GPR56, and the protein can

be easily detected by Western blot. Either the GD7 preimmune or 1st bleed was incubated with A2058/GPR56 at 1:100 dilution. Two days later, cells are harvested, and protein lysates were made. Western blotting was conducted with GD7 antibody, and the data is shown in Figure 32. Decreased levels of GPR56 were observed after the cells were incubated with the antibody. This finding is consistent with target down-regulation mediated by GPR56 siRNA.

In summary, the results above show that GPR56 antibody can down-regulate GPR56 protein level, consistent with siRNA effect. The antibody also reduced tumor cell growth, and induces apoptosis preferentially in tumor cells compared to normal cells.

Example 2

1. General

DNER is a type I transmembrane protein. It has ten extracellular repeats homologous to the epidermal growth factor and a short cytoplasmic tail with tyrosine-based and dileucine-type sorting motifs. The tyrosine-based motif binds to the adaptor binding complex AP-1 and targets DNER to the dendritic plasma membrane of neurons. Eiraku et al., J. Biol. Chem., 277:25400-25407 (2002). The motifs of DNER are summarized in Table 4 below:

Table 4 – DNER Domains

Name	Begin	End	E-value
<u>Signal peptide</u>	1	29	-
<u>EGF like</u>	48	92	3.80e-05
<u>EGF like</u>	96	133	5.40e-06
<u>Low Complexity</u>	203	213	-
<u>EGF like</u>	306	348	1.70e+01
<u>EGF like</u>	349	390	7.60e-07
<u>EGF CA</u>	392	428	3.50e-08
<u>EGF like</u>	430	466	2.10e-06
<u>EGF like</u>	469	503	5.30e-07
<u>EGF CA</u>	505	541	1.20e-09
<u>EGF like</u>	544	579	4.80e-04
<u>FOLN</u>	546	568	4.20e-02
<u>EGF CA</u>	581	617	3.30e-12
<u>Transmembrane</u>	639	661	-

2. Over-expression of DNER is associated with cell transformation

Cellular transformation is often the result of activation of oncogenes or inactivation of tumor suppressor genes, whereas transformation reversion could result from activation of tumor suppressors or inactivation of oncogenes. Frequently, activation and inactivation of gene functions can be reflected in changes of gene expression. Global gene expression profiling such as DNA chip analysis is the most powerful approach in revealing changes in gene expression. The Affymetrix GeneChip DNA microarray (U138 A-chip) was used to analyze the HeLa and HeLaHF cell pair to identify genes that are significantly altered in their relative expression levels. The expression of hundreds of genes is affected in the HeLa/HF system, with changes up to 250 fold. Focus was on genes that are downregulated in HeLaHF cells as possible oncogenes, which can be evaluated as potential drug targets. Among the down-regulated genes, "DNER," or Delta/Notch-like epidermal growth factor (EGF)-related receptor (Accession #: NM_139072) (HE60; SEQ ID NO:3) was identified. This downregulation was also confirmed by real time PCR (Figure 33). These results show potential oncogenic properties for this gene and the encoded protein.

3. Expression of DNER in cancer cells

In normal tissue, DNER is specifically expressed in the brain (Eiraku et al., *J. Biol. Chem.*, 277:25400-25407 (2002) and Figure 10). In addition, DNER is expressed in several cancer tissues including cancers of bone, cervix, head and neck, lung and lymph nodes (Figure 10).

4. siRNA knockdown of DNER in cancer cell lines

Two DNER-specific siRNAs and a control siRNA were transiently transfected into various cell lines using OligofectamineTM following the manufacturer's instructions (Invitrogen, Carlsbad, CA). The following cell lines were used to down-regulate the expression of DNER (SEQ ID NO:2): the cervical cancer cell line HeLa; the lung cancer cell lines HOP62, HOP92, H460 and A549; PC-3 (prostate); and A2058 (melanoma).

siRNAs were prepared using a GeneSilencerTM siRNA Construction kit following the manufacturer's instructions (Ambion). The sense strand of the first

siRNA (labeled “2”) was CGUCAGCUGUCUGAACGGAUU (SEQ ID NO:10), and the antisense strand was UCCGUUCAGACAGCUGACGUU (SEQ ID NO:11). The sense strand of the second siRNA (labeled “4”) was GGCUAUGAAGGUCCCAACUUU (SEQ ID NO:12), and the antisense strand was AGUUGGGACCUUCAUAGCCUU (SEQ ID NO:13).

5. Validation Assays

Transfected cells were trypsinized and plated into 96-well plates. The effect of the knock-down of DNER by siRNAs on cell growth was assessed using procedures substantially similar to those described in Example 1 for GPR56.

a. Anchorage-dependent growth

Cell growth in liquid culture was quantified on day 1 and day 3 (or day 4) by AlamarBlue™ staining. The results for the cell lines HOP92, H460, A549 and HOP62 are shown in Figures 11A-D, respectively. Both DNER-specific siRNAs caused a significant reduction of anchorage-dependent growth of HOP92 cells and A549 cells. Growth of H460 cells was inhibited by one (“4”) of the two DNER-specific siRNAs, and growth of HOP62 cells was moderately affected by both DNER-specific siRNAs.

b. Anchorage-independent growth

Cell growth in soft-agar culture was quantified on day 1 and day 7 (or day 8) by AlamarBlue™ staining. The results for the cell lines HeLa, H460, A549 and HOP62 are shown in Figures 12A-D, respectively. Both DNER-specific siRNAs caused a significant reduction of anchorage-independent growth in HeLa cells, A549 cells and H460 cells. Growth of HOP62 cells was also significantly reduced by one DNER-specific siRNA (“4”), while the other (“2”) had a moderate effect. Additonla results are shown in Table 5 below.

Table 5 - Transient transfection of cancer cells with *in vitro* synthesized HE60 (DNER) targeting siRNAs. NSCL= non small cell lung cancer.

Cancer	Cell Line	% Phenotype*		Taqman (mRNA levels)
		Liquid culture	Soft agar culture	
NSCL	Hop62	10-15%	20-60%	60-80%
NSCL	H460	30%	40-50%	30%
NSCL	Hop92	40%	No data	40-90%

*Range for % phenotype indicates levels obtained with different siRNA

5 In addition, lentiviral shRNA vectors (pSD31) (Ke et al., 2004) against HE60 were used to transduce the following cancer cell lines: non-small cell lung cancer (NSCL) Hop62 cells, melanoma line A2058 cells and prostate cancer PC3 cells. The stable cell lines were generated by selection using puromycin, and they were evaluated for cell growth under both anchorage-dependent and anchorage-
10 independent conditions. Overall, a modest reduction was observed in anchorage-dependent growth of cells silenced for HE60 (DNER) (Figures 34A, 35A, 36A and 37A), while a significant reduction was observed for and a pronounced reduction was observed in anchorage-independent growth (soft agar; Figures 34B, 35B, 36B and 37B). The melanoma cell line A2058 was the only cell line which showed an
15 anchorage dependent (liquid culture) phenotype.

c. Down-regulation of DNER mRNA

mRNA was extracted from the cell lines HeLa, HOP92, H460 and HOP62 transiently transfected with one of the DNER-specific siRNAs or with control siRNA and subject to real-time RT-PCR (Taqman®) analysis. Figures 13A-D show that both
20 DNER-specific siRNAs caused a reduction of DNER mRNA levels in HeLa, HOP92 and HOP62. siRNA "4" also reduced the DNER mRNA level in H460 cells. These findings explain the biological phenotype observed in the previous two experiments (reduction in anchorage-dependent growth in liquid culture and reduction in
25 anchorage-independent growth in soft-agar).

6. Xenograft models

Anchorage-independent growth *in vitro* has been shown to correlate well with the cancer cell tumorigenicity *in vivo*. Since the HE60 (DNER) silencing caused significant reduction of cancer cell anchorage-independent growth, the effect of HE60
5 silencing on the tumorigenicity of cancer cells was examined. The PC3 prostate cancer xenograft model was used. Lentiviral vectors pSD31-shHE60 and pSD31-shCNTL transduced PC3 cells were injected into nude mice, and the ability of these cells to form tumors was monitored. As shown in Figure 38, a significant reduction of the tumorigenicity of pSD31-shHE60 PC3 tumors was observed as compared with
10 the pSD31-shCNTL transduced PC3 tumors. These results show that silencing DNER can significantly the reduce tumorigenicity of cancer cells *in vivo*.

7. DNER antibodies

Antibodies specific for DNER are made in the same way as described for the
15 GPR56 antibodies in Example 2, using the domains described in Table 4 above. These antibodies are useful for slowing or reducing the growth of the cells lines described above in Example 2.

All references made herein, including articles, patent applications and any other publications, are incorporated by reference in their entirety.

We claim:

1. A method of inhibiting cancer cells in a subject, comprising administering to said subject a molecule that binds to an amino acid sequence comprising SEQ ID
5 NO:2; SEQ ID NO:2 wherein position 306 is histidine; a domain thereof; or a nucleic acid sequence encoding said amino acid sequence, thereby decreasing the expression or activity of said amino or nucleic acid sequence.
2. The method of claim 1, wherein said molecule is an siRNA.
10
3. The method of claim 2, wherein said siRNA is SEQ ID NOS:5 or 6.
4. The method of claim 1, wherein said molecule is an antibody.
- 15 5. The method of claim 4, wherein said antibody is GD3, GD5 or GD 7.
6. The method of claim 1, wherein said molecule is a small organic molecule.
7. The method of claim 1, wherein said cancer is selected from the group
20 consisting of melanoma, cervical cancer, colon cancer, prostate cancer, lung cancer, ovarian cancer, pancreatic cancer, breast cancer, astrocytoma and glioma.
8. The method of claim 7, wherein said cancer is melanoma.
- 25 9. A method of inhibiting cancer cells in a subject, comprising administering to said subject a molecule that binds to an amino acid sequence comprising SEQ ID NO:3 or a domain thereof, or a nucleic acid sequence encoding said amino acid sequence, thereby decreasing the expression or activity of said amino or nucleic acid
30 sequence.
10. The method of claim 9, wherein said molecule is an siRNA.
11. The method of claim 10, wherein said siRNA is SEQ ID NOS:10 or 12.

12. The method of claim 9, wherein said molecule is an antibody.

13. The method of claim 9, wherein said molecule is a small organic molecule.

5 14. The method of claim 1, wherein said cancer is selected from the group consisting of melanoma, cervical cancer, colon cancer, prostate cancer, lung cancer, ovarian cancer, breast cancer and glioma.

15. An anti-GPR56 antibody.

10

16. The antibody of claim 15, selected from the group consisting of GD3, GD4, GD5, GD6, GD7 and GD8.

17. A method of identifying an agent that inhibits cancer, comprising:

15

a. introducing said agent into cancer cells or in touch with cancer cells, wherein said agent binds to a molecule encoding SEQ ID NO:2, or encoding SEQ ID NO:2 with histidine at position 306 or comprising the RNA correlate of said molecules; and

20 b. measuring the level of inhibition of said cells, where an increase in level indicates said agent inhibits cancer.

18. The method of claim 17, wherein said molecule comprises SEQ ID NO:1.

25 19. The method of claim 17, wherein said molecule comprises the RNA correlate of SEQ ID NO:1.

20. The method of claim 17, wherein said molecule consists of SEQ ID NO:1.

30 21. The method of claim 17, wherein said inhibition is measured by an apoptosis assay, where an increase in the level of apoptosis indicates that said agent inhibits cancer.

22. The method of claim 17, wherein said inhibition is measured by a proliferation assay, where a decrease in the rate of cell division indicates that said agent inhibits cancer.

5 23. The method of claim 17, wherein said inhibition is measured by a cell adhesion assay.

24. The method of claim 17, wherein said inhibition is measured by a reporter assay.

10

25. The method of claim 17, wherein said inhibition is measured by *in vivo* xenograft tumor growth.

15 26. The method of claim 17, wherein said cancer is selected from the group consisting of melanoma, cervical cancer, colon cancer, prostate cancer, lung cancer, ovarian cancer, pancreatic cancer, breast cancer and glioma.

27. The method of claim 17, wherein said cancer is melanoma.

20 28. A method of identifying an agent that inhibits cancer, comprising:

a. introducing said agent into cancer cells, wherein said agent binds to a molecule comprising SEQ ID NO:2 or SEQ ID NO:2 wherein position 306 is histidine; and

25 b. measuring the level of inhibition of said cells, where an increase in level indicates said agent inhibits cancer.

29. The method of claim 28, wherein said molecule comprises SEQ ID NO:2.

30 30. The method of claim 28, wherein said molecule comprises SEQ ID NO:2 wherein position 306 is histidine.

31. The method of claim 28, wherein said molecule consists of SEQ ID NO:2.

32. The method of claim 28, wherein said inhibition is measured by an apoptosis assay, where an increase in the level of apoptosis indicates that said agent inhibits cancer.

5 33. The method of claim 28, wherein said inhibition is measured by a proliferation assay, where a decrease in the rate of cell division indicates that said agent inhibits cancer.

34. The method of claim 28, wherein said cancer is selected from the group
10 consisting of melanoma, cervical cancer, colon cancer, prostate cancer, lung cancer, ovarian cancer, breast cancer and glioma.

35. The method of claim 28, wherein said cancer is melanoma.

15 36. A method of identifying an agent that inhibits cancer, comprising:
a. introducing said agent into cancer cells, wherein said agent binds to a compound comprising an amino acid sequence selected from the group consisting of amino acid 1 to amino acid 26 of SEQ ID NO:2, amino acid 342 to amino acid 394 of SEQ ID NO:2, amino acid 405 to amino acid 427 of SEQ ID NO:2, amino acid 447 to
20 amino acid 469 of SEQ ID NO:2, amino acid 479 to amino acid 501 of SEQ ID NO:2, amino acid 514 to amino acid 536 of SEQ ID NO:2, amino acid 575 to amino acid 597 of SEQ ID NO:2, amino acid 609 to amino acid 631 of SEQ ID NO:2, and amino acid 637 to amino acid 659 of SEQ ID NO:2; and
b. measuring the level of inhibition of said cells, where an increase in level
25 indicates said molecule inhibits cancer.

37. A method of identifying an agent that inhibits cancer, comprising:
a. introducing said agent into cancer cells, wherein said agent binds to a molecule comprising SEQ ID NO:3 or comprising the RNA correlate of SEQ ID
30 NO:3; and
b. measuring the level of inhibition of said cells, where an increase in level indicates said agent inhibits cancer.

38. The method of claim 37, wherein said molecule comprises SEQ ID NO:3.

39. The method of claim 37, wherein said molecule comprises the RNA correlate of SEQ ID NO:3.

40. The method of claim 38, wherein said molecule consists of SEQ ID NO:3.

5

41. The method of claim 37, wherein said inhibition is measured by an apoptosis assay, where an increase in the level of apoptosis indicates that said agent inhibits cancer.

10 42. The method of claim 37, wherein said inhibition is measured by a proliferation assay, where a decrease in the rate of cell division indicates that said agent inhibits cancer.

15 43. The method of claim 37, wherein said cancer is selected from the group consisting of melanoma, cervical cancer, colon cancer, prostate cancer, lung cancer, ovarian cancer, breast cancer and glioma.

44. The method of claim 37, wherein said cancer is melanoma.

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ATGACTCCCCAGTCGCTGCTGCAGACGACACTGTTCCCTGCTGAGTCTGCTCTTCCCTGGTCCAAGGTGCCC
ACGGCAGGGGCCACAGGGAAGACTTTTCGCTTCTGCAGCCAGCGGAACCAGACACACAGGAGCAGCCTCCA
CTACAAACCCACACCAGACCTGCGCATCTCCATCGAGAACTCCGAAGAGGCCCTCACAGTCCATGCCCCCT
TTCCCTGCAGCCCACCCTGCTTCCCGATCCTTCCCTGACCCCAGGGGCCTCTACCACTTCTGCCTCTACT
GGAACCGACATGCTGGGAGATTACATCTTCTCTATGGCAAGCGTGACTTCTTGCTGAGTGACAAAGCCTC
TAGCCTCCTCTGCTTCCAGCACCAGGAGGAGAGCCTGGCTCAGGGCCCCCGCTGTTAGCCACTTCTGTCTC
ACCTCCTGGTGGAGCCCTCAGAACATCAGCCTGCCCAGTGCCGCCAGCTTACCTTCTCCTTCCACAGTCTC
TCCCCACACGGCCGCTCACAATGCCTCGGTGGACATGTGCGAGCTCAAAGGGACCTCCAGCTGCTCAG
CCAGTTCTGAAGCATCCCCAGAAGGCCTCAAGGAGGCCCTCGGCTGCCCCCGCCAGCCAGCAGTTGCAG
AGCCTGGAGTCGAAACTGACCTCTGTGAGATTTCATGGGGGACATGGTGTCTTTCGAGGAGGACCGGATCA
ACGCCACGGTGTGGAAGCTCCAGCCCACAGCCGGCCTCCAGGACCTGCACATCCACTCCCGGCAGGAGGA
GGAGCAGAGCGAGATCATGGAGTACTCGGTGCTGCTGCCTCGAACACTCTTCCAGAGGACGAAAGGCCGG
AGCGGGGAGGCTGAGAAGAGACTCCTCCTGGTGGACTTCAGCAGCCAAGCCCTGTTCCAGGACAAGAATT
CCAGCCAAGTCTTGGGTGAGAAGGTCTTGGGGATTGTGGTACAGAACACCAAAGTAGCCAACCTCACGGA
GCCCGTGGTGTCTCACTTTCCAGCACCAGCTACAGCCGAAGAATGTGACTCTGCAATGTGTGTTCTGGGTT
GAAGACCCACATTGAGCAGCCCCGGGGCATTTGGAGCAGTGCTGGGTGTGAGACCGTCAGGAGAGAAAACC
AAACATCCTGCTTCTGCAACCACTTGACCTACTTTGCAGTGCTGATGGTCTCCTCGGTGGAGGTGGACGC
CGTGACACAAGCACTACCTGAGCCTCCTCTCCTACGTGGGCTGTGTCTGCTCTGCCCCTGGCCTGCCTTGTCT
ACCATTGCCGCCTACCTCTGCTCCAGGGTGCCCCCTGCCGTGCAGGAGGAAACCTCGGGACTACACCATCA
AGGTGCACATGAACCTGCTGCTGGCCGTCTTCTGCTGGACACGAGCTTCTGCTCAGCGAGCCGGTGGC
CCTGACAGGCTCTGAGGCTGGCTGCCGAGCCAGTGCCATCTTCTGCACTTCTCCCTGCTCACCTGCCTT
TCCTGGATGGGCCTCGAGGGGTACAACCTCTACCGACTCGTGGTGGAGGTCTTTGGCACCTATGTCCCTG
GCTACCTACTCAAGCTGAGCGCCATGGGCTGGGGCTTCCCCATCTTTCTGGTGACGCTGGTGGCCCTGGT
GGATGTGGACAACTATGGCCCCATCATCTTGGCTGTGCATAGGACTCCAGAGGGCGTCATCTACCTTCC
ATGTGCTGGATCCGGGACTCCCTGGTCAGCTACATCACCAACCTGGGCCTCTTCAGCCTGGTGTCTTCTGT
TCAACATGGCCATGCTAGCCACCATGGTGGTGCAGATCCTGCGGCTGCGCCCCACACCCAAAAGTGGTC
ACATGTGCTGACACTGCTGGGCCTCAGCCTGGTCTTGGCCTGCCCTGGGCCTTGATCTTCTTCTCCTTT
GCTTCTGGCACCTTCCAGCTTGTCTGCTCTACCTTTTTCAGCATCATCACCTCCTTCCAAGGCTTCTCTCA
TCTTCATCTGGTACTGGTCCATGCGGCTGCAGGCCCGGGGTGGCCCCCTCCCCTCTGAAGAGCAACTCAGA
CAGCGCCAGGCTCCCCATCAGCTCGGGCAGCACCCTCGTCCAGCCGCATCTAG

Figure 1 (SEQ ID No:1)

MTPQSLQLQTTLFLLSLLFLVQGAHGRGHREDFRFCQQRNQTHRSSLHYKPTPDLRISIENSEEALTVHAP
FPAAHPASRSFDPDPRGLYHFCLYWNRHAGRLHLLYGKRDFLSDKASSLLCFQHQQEESLAQGPPLIATSV
TSWWSPQNISLPSAASFTFSFHSPHTAAHNASVDMCELKRDLQLLSQFLKHPQKASRRPSAAPASQQLQ
SLESKLTSVRFMGDMVSFEEDRINATVWKLQPTAGLQDLHIHSRQEEEQSEIMEYSVLLPRTLQRTKGR
SGEAEKRLLLVDFSSQALFQDKNSSQVLGEKVLGIVVQNTKVANLTEPVVLTQHQQLQPKNVTLCVFWV
EDPTLSSPGHWSSAGCETVRRETQTSCFCNHLTYFAVLMVSSVEVDVHKHYLSLLSYVGCVVVSALACLV
TIAAYLCSRVPPLPCRRKPRDYTIKVHNMNLLAVFLDTSFLLSEPVALTGSEAGCRASAIFLHFSLLTCL
SWMGLEGYNLYRLVVEVFGTYVPGYLLKLSAMGWGFPIFLVTLVALVDVDNYGPIILAVHRTPEGVIYPS
MCWIRDSLVSYITNLGLFSLVFLFNMAMLATMVVQILRLRPHTQKWSHVLTLGLSLVLGLPWALIFFSF
ASGTFQLVVLVLYLFSIITSFQGFLLFIWYWSMRLQARGGPSPLKSNSDSARLPISSGSTSSRI

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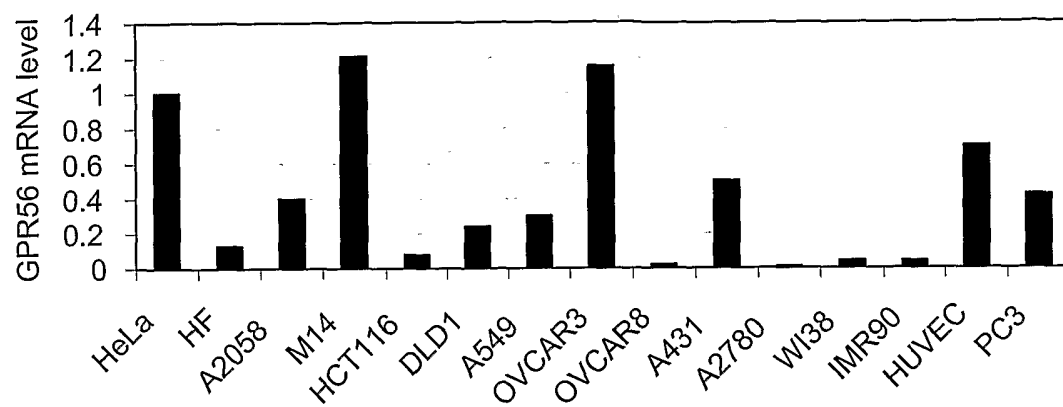


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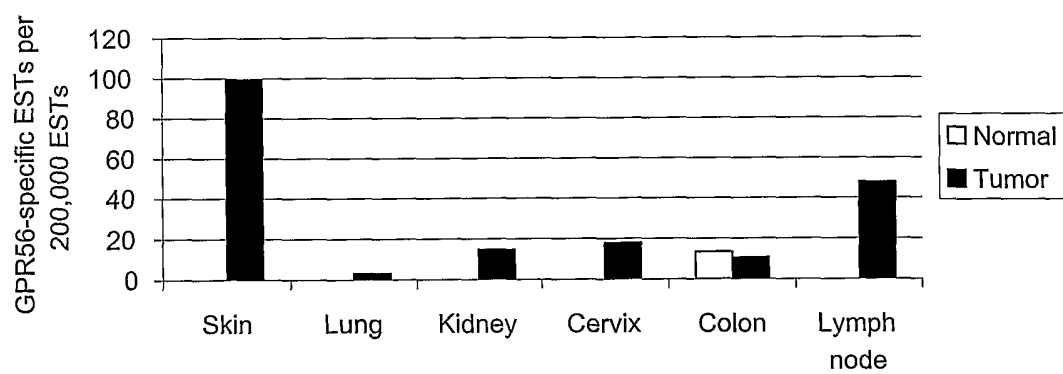


Figure 3B

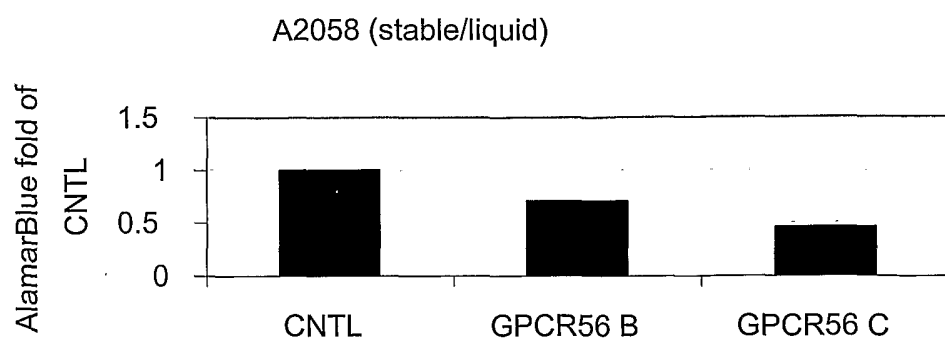


Figure 4A

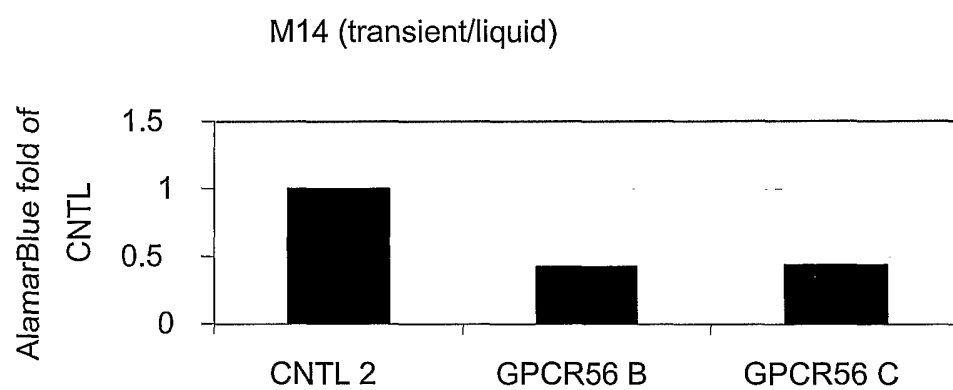


Figure 4B

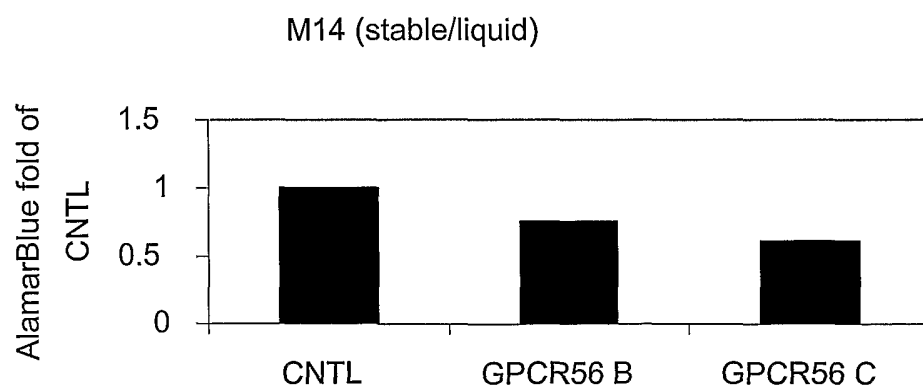


Figure 4C

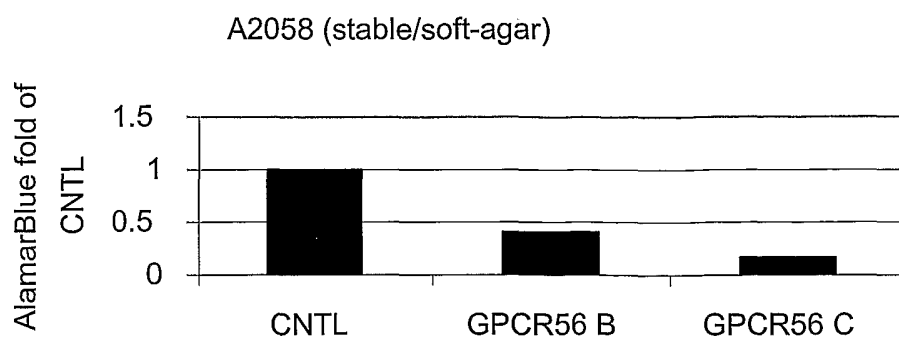


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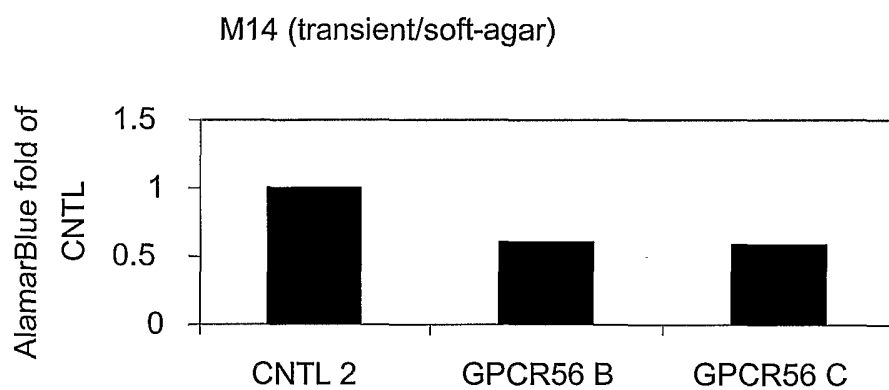


Figure 5B

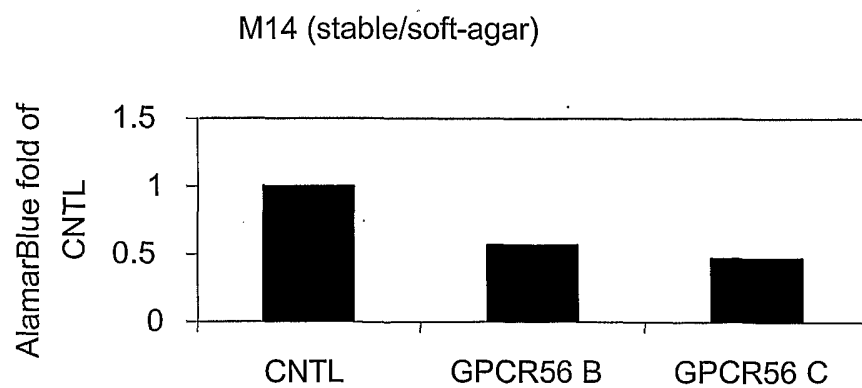


Figure 5C

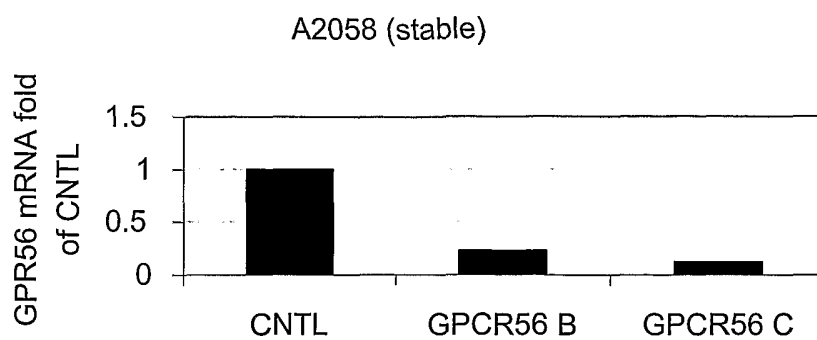


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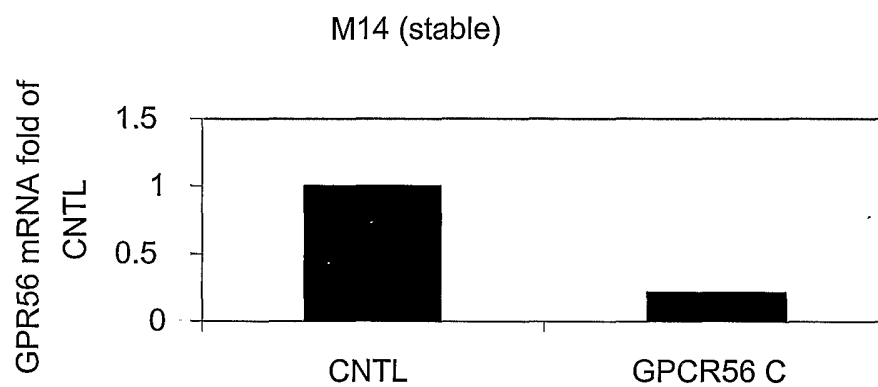


Figure 6B

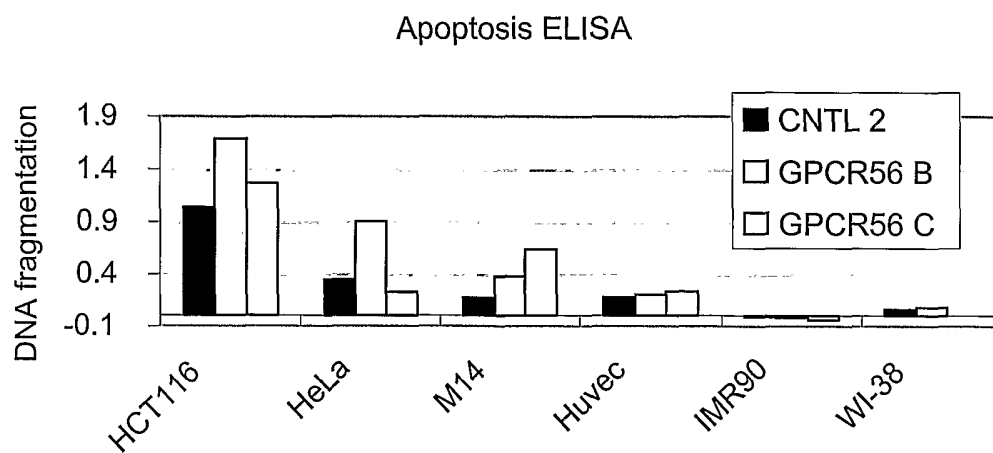


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Figure 8 (SEQ ID NO:3)

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Figure 9 (SEQ ID NO:4)

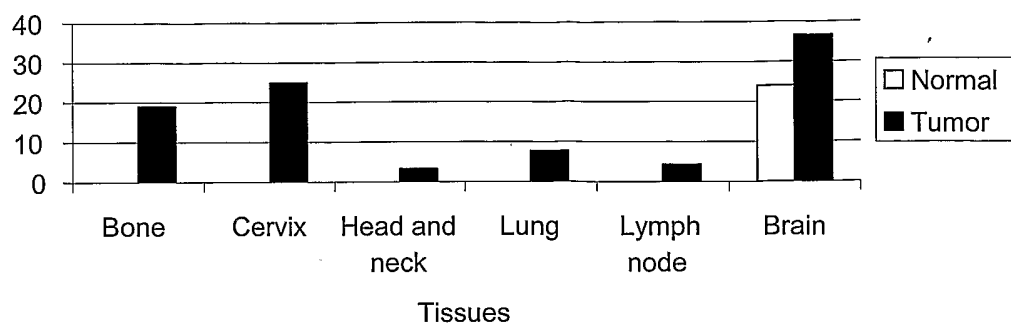


Figure 10

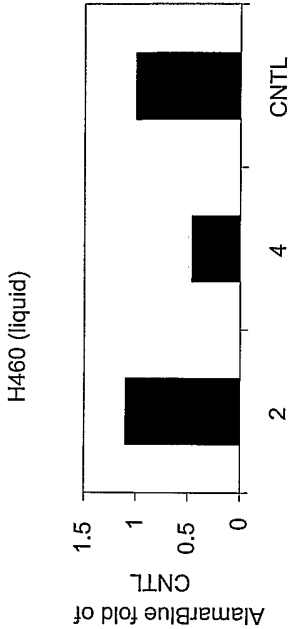


Figure 11B

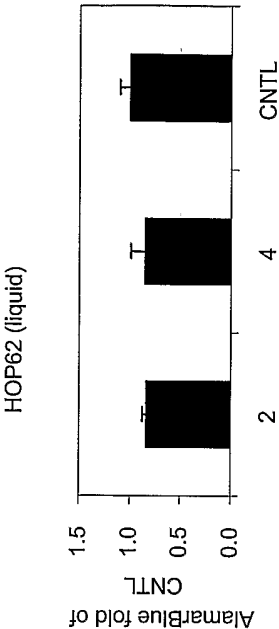


Figure 11D

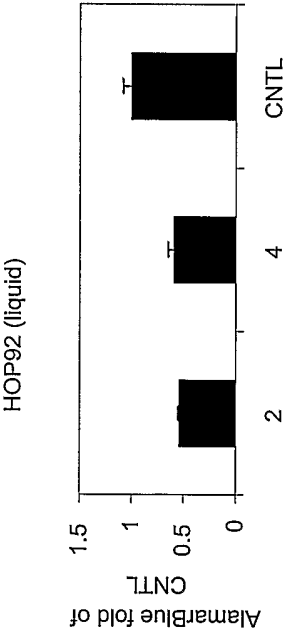


Figure 11A

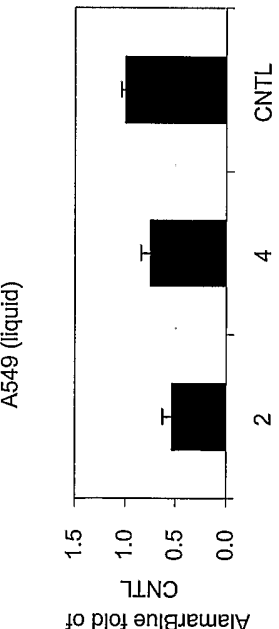


Figure 11C

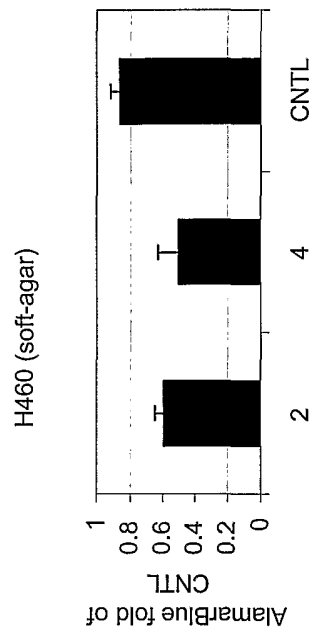


Figure 12A

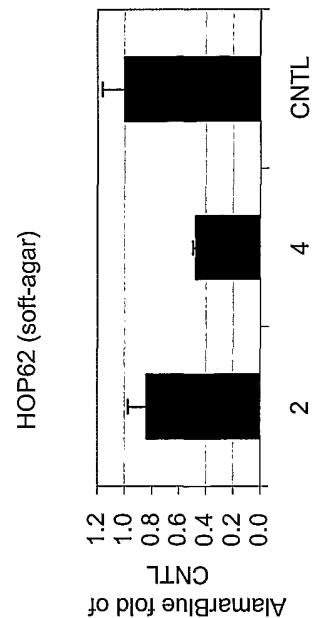


Figure 12B

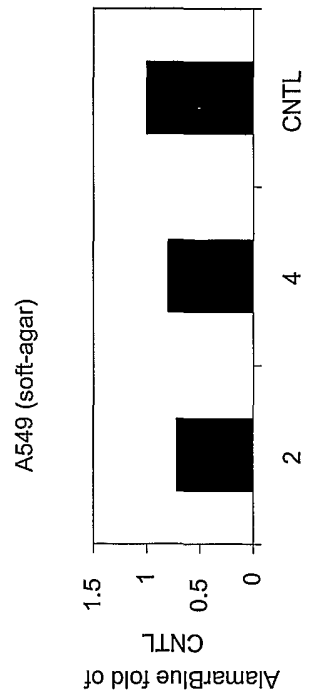


Figure 12C

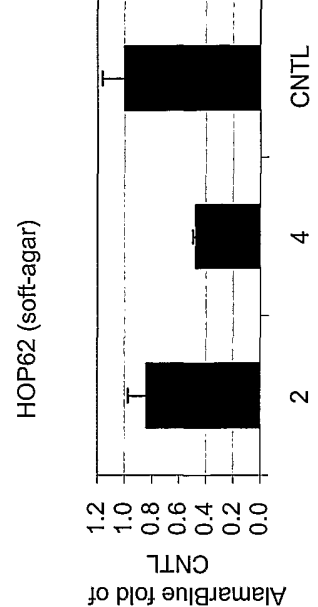


Figure 12D

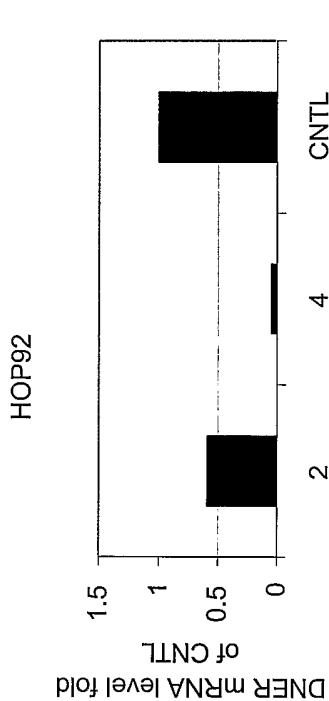


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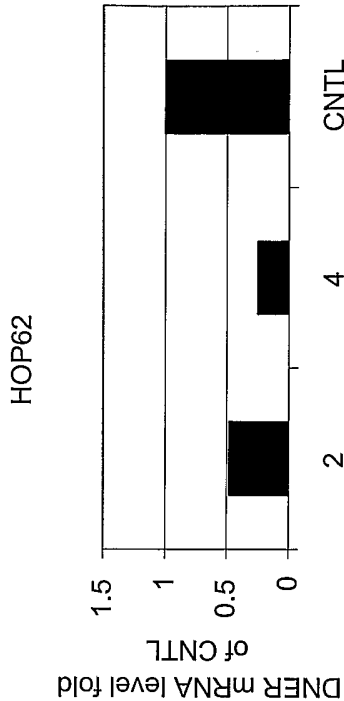


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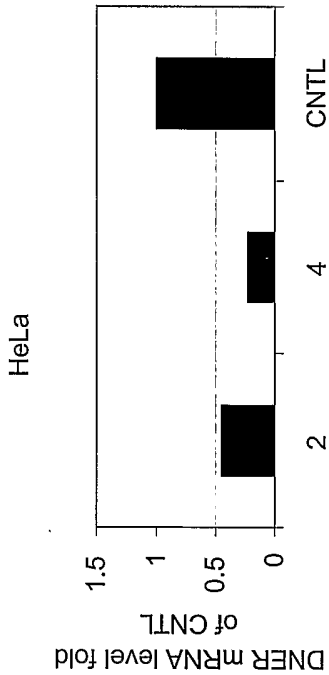


Figure 13C

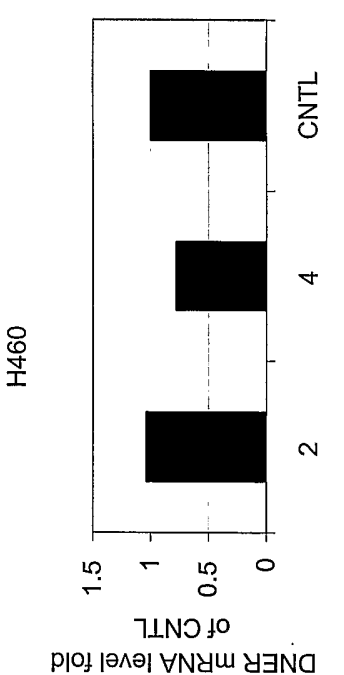


Figure 13D

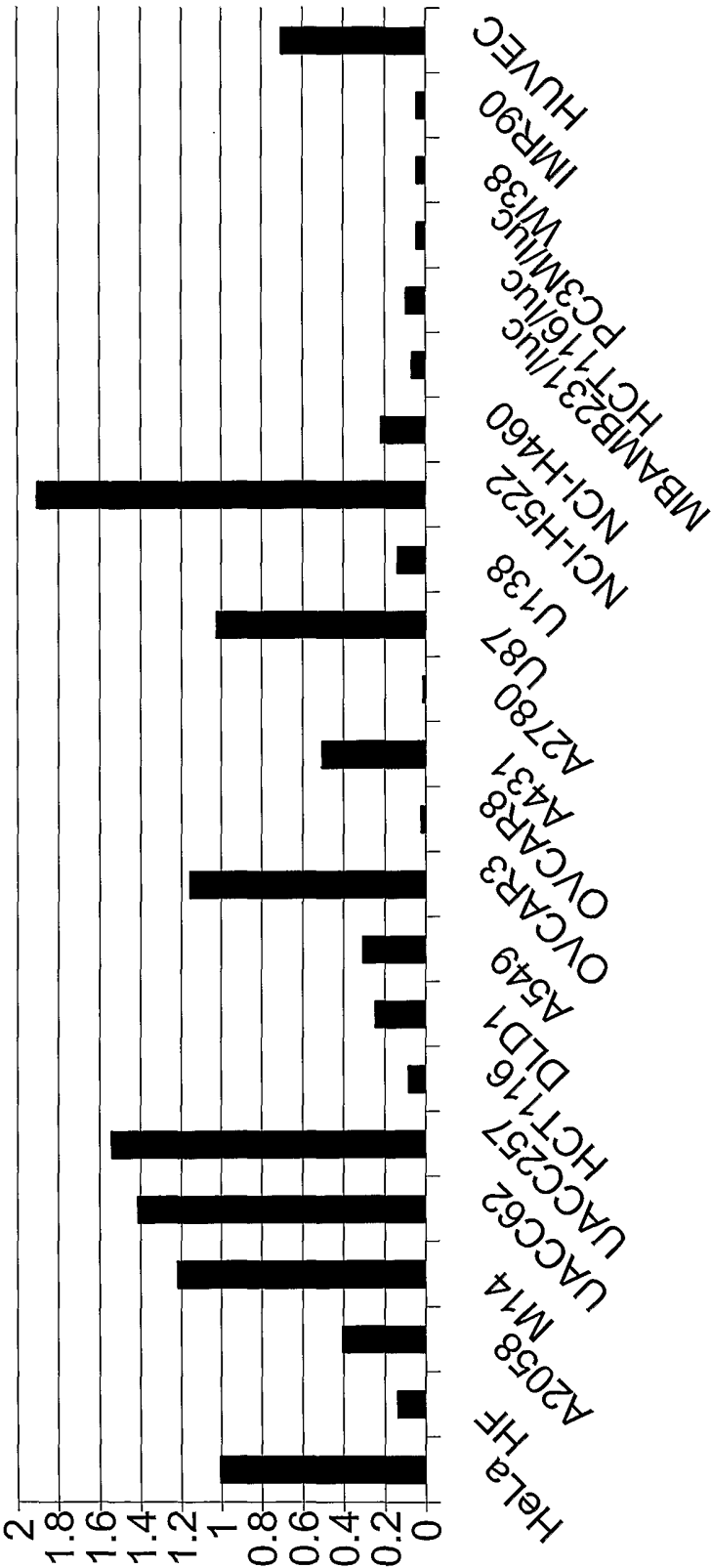


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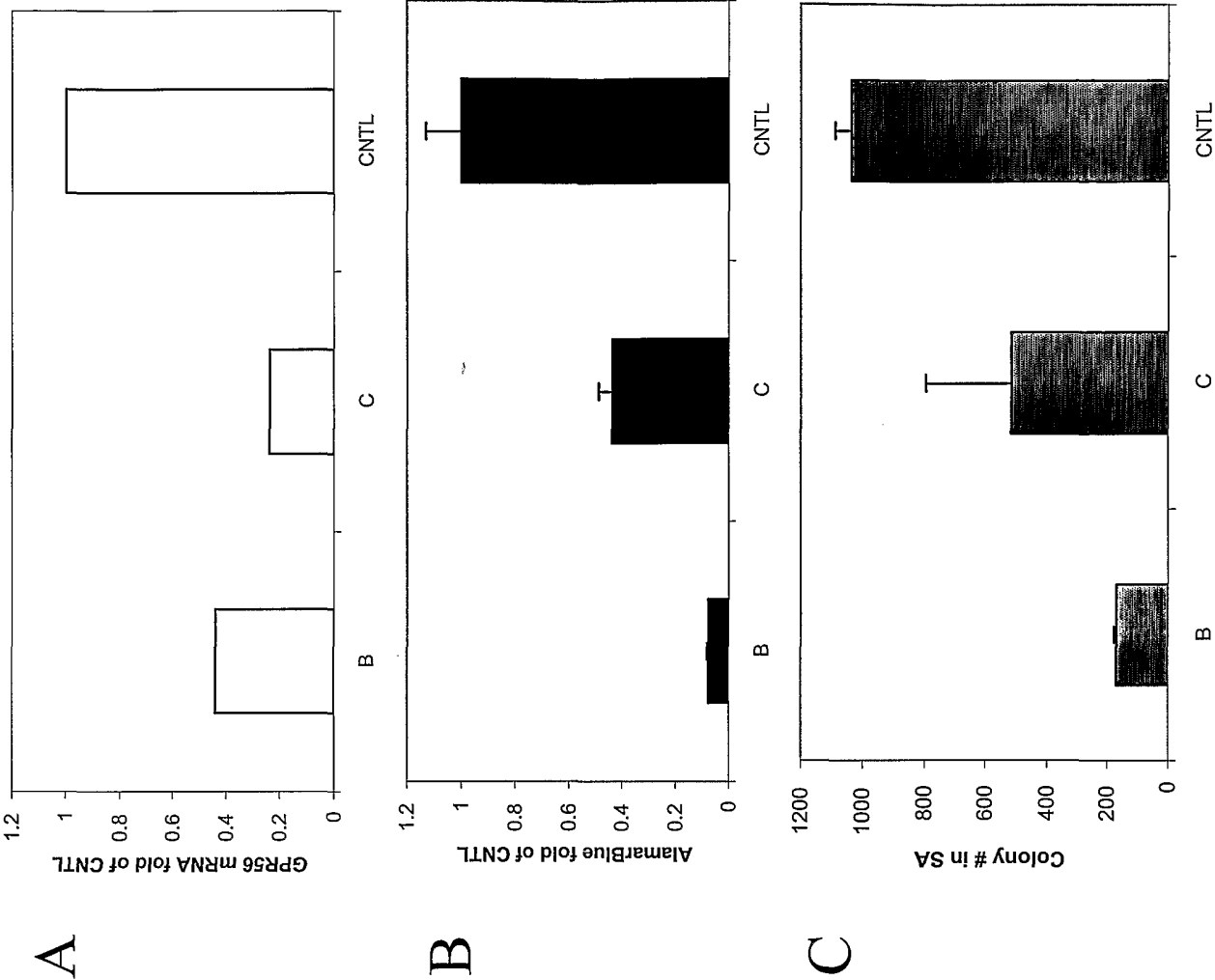


Figure 15 HeLa

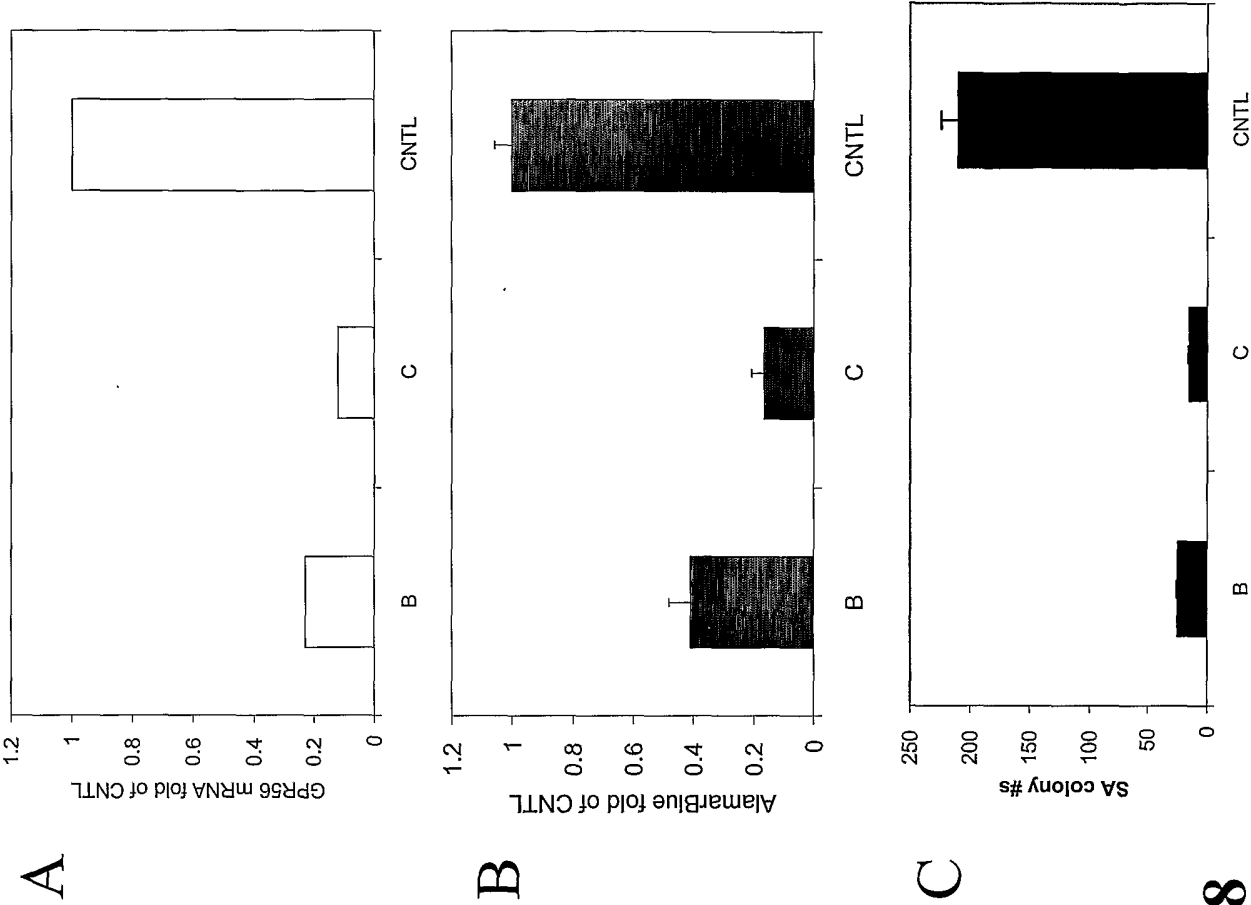


Figure 16 A2058

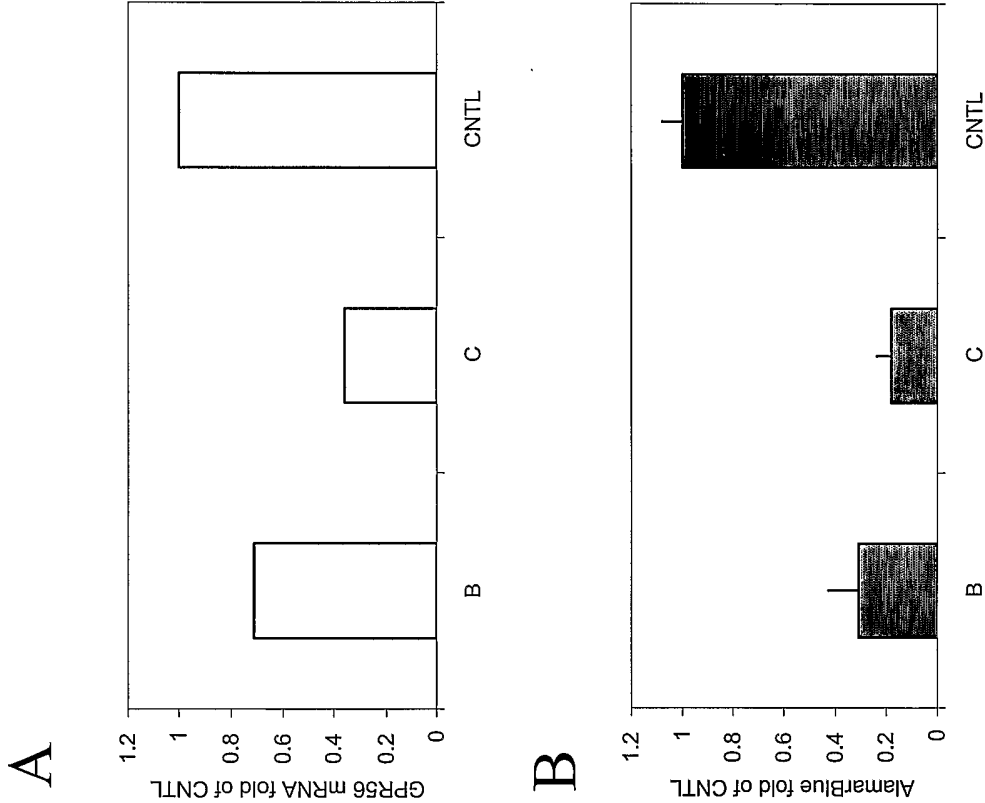


Figure 17 PC3

FIGURE 18A OVCAR 8

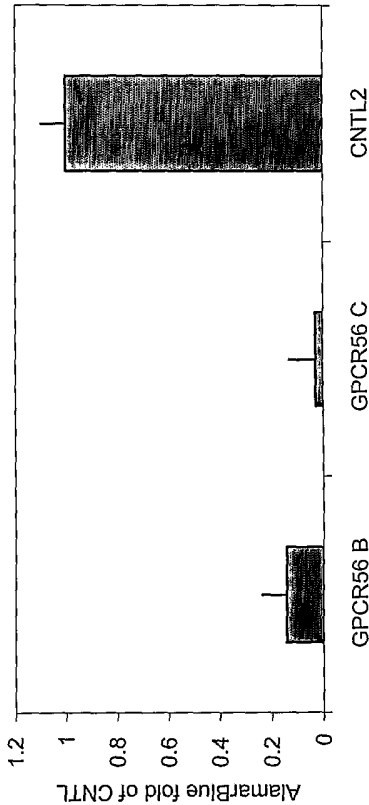


FIGURE 18B OVCAR 3

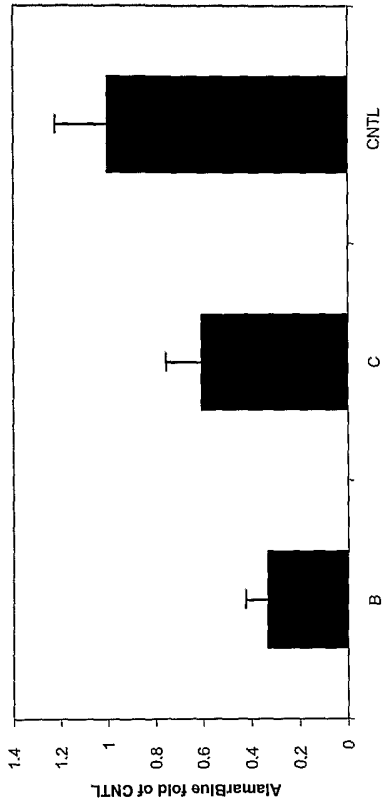


FIGURE 19A AsPC1

FIGURE 19B

NCI-H460

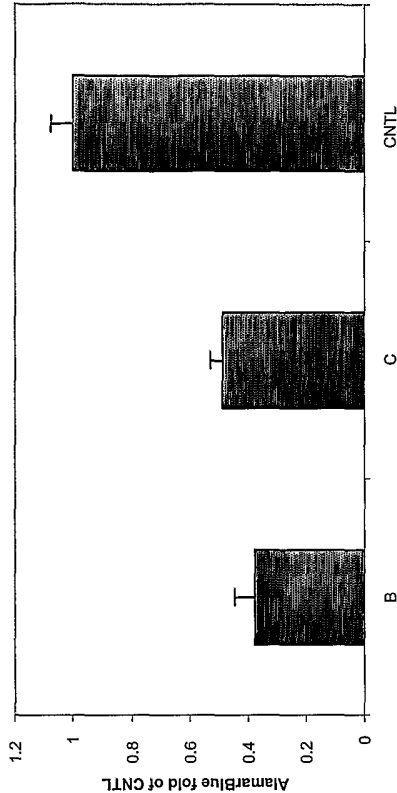
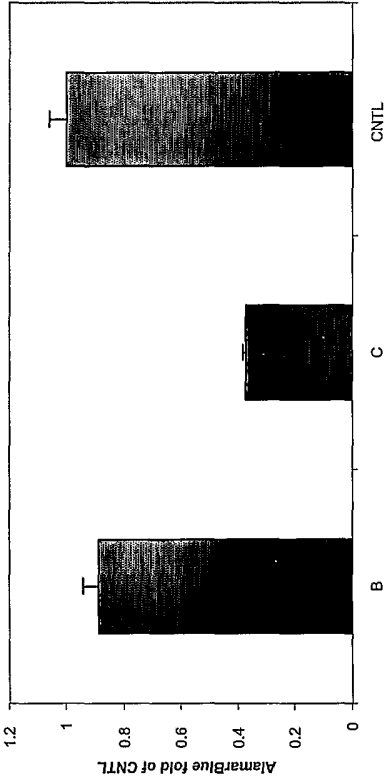
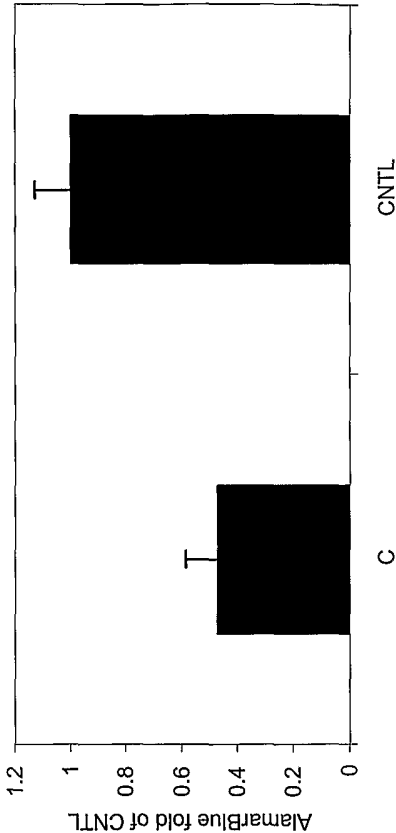


FIGURE 19C M14



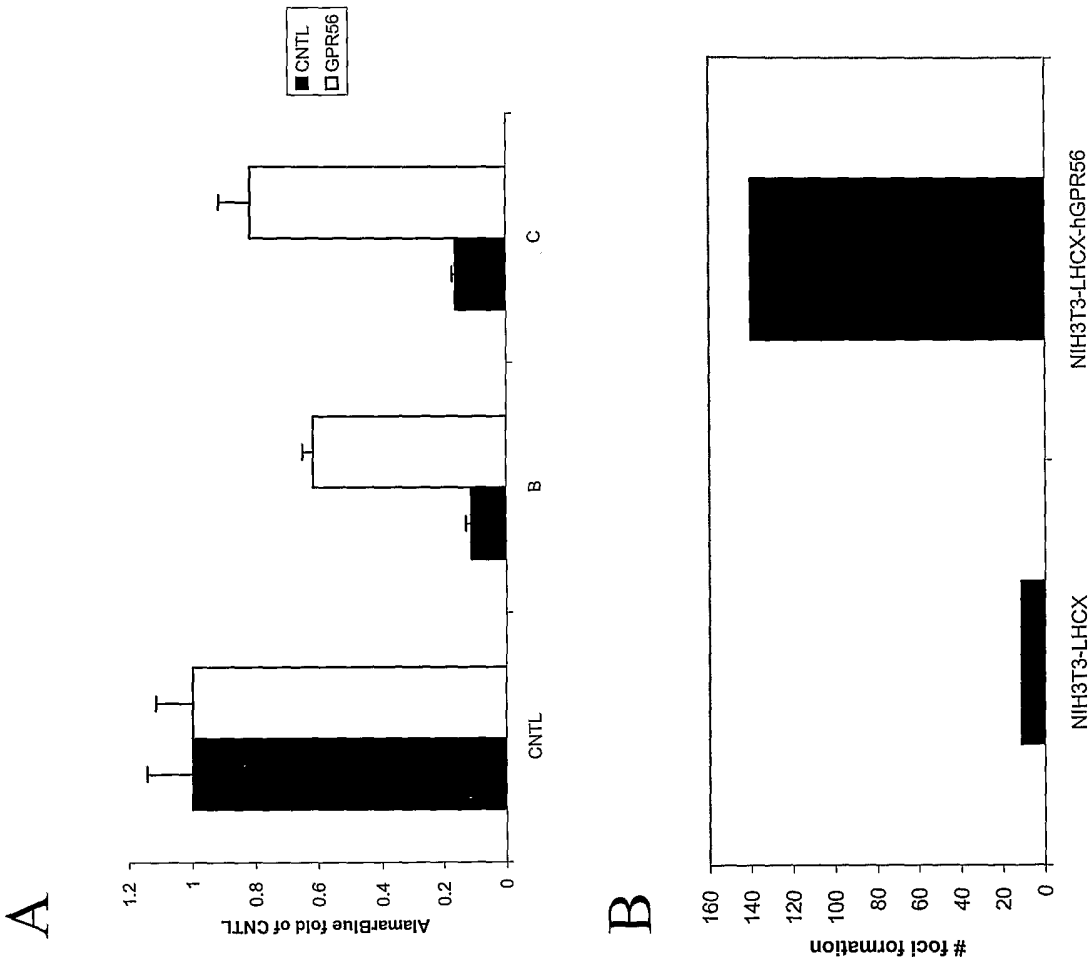


Figure 20 cDNA over-expression

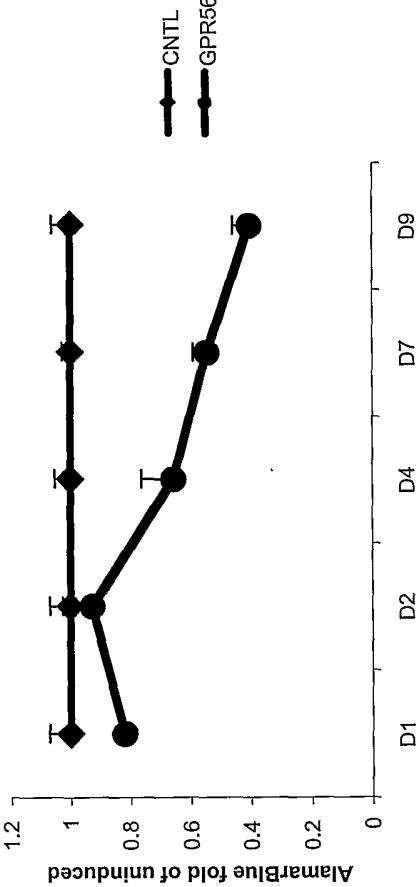
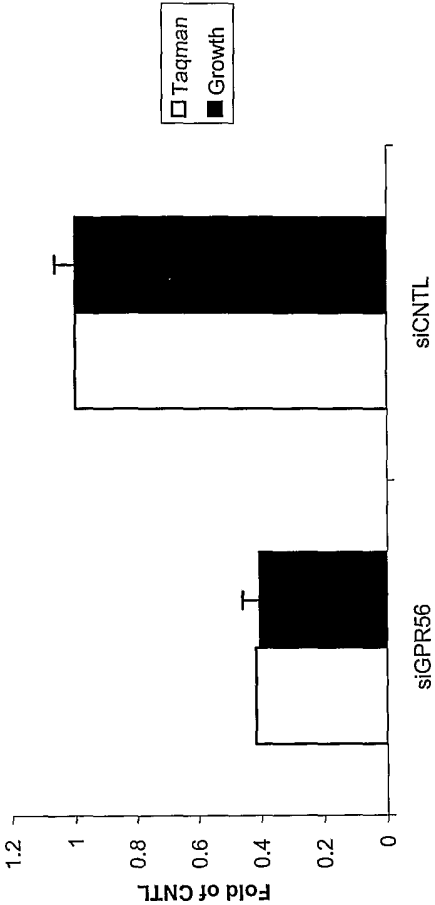


Figure 21

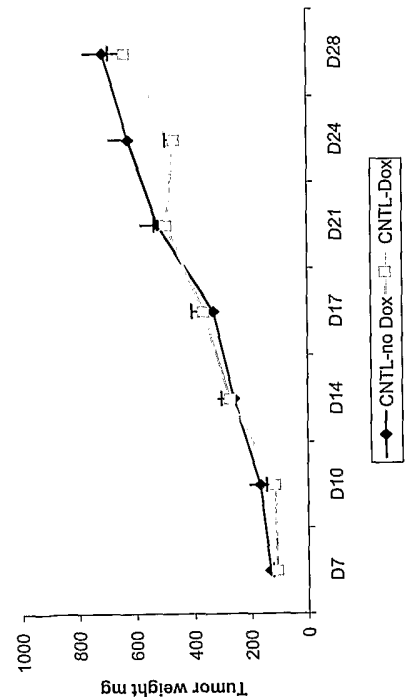
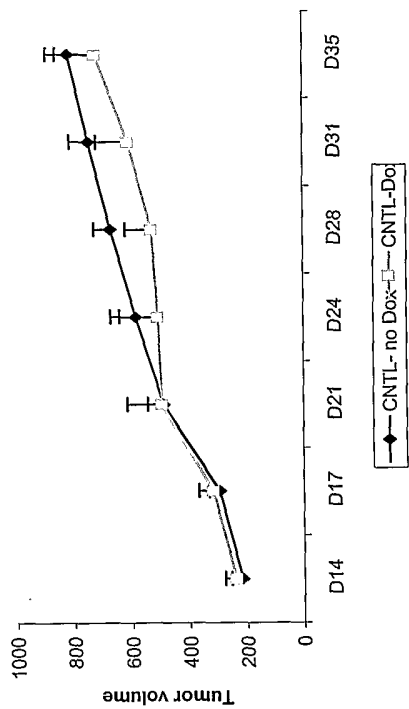


Figure 22

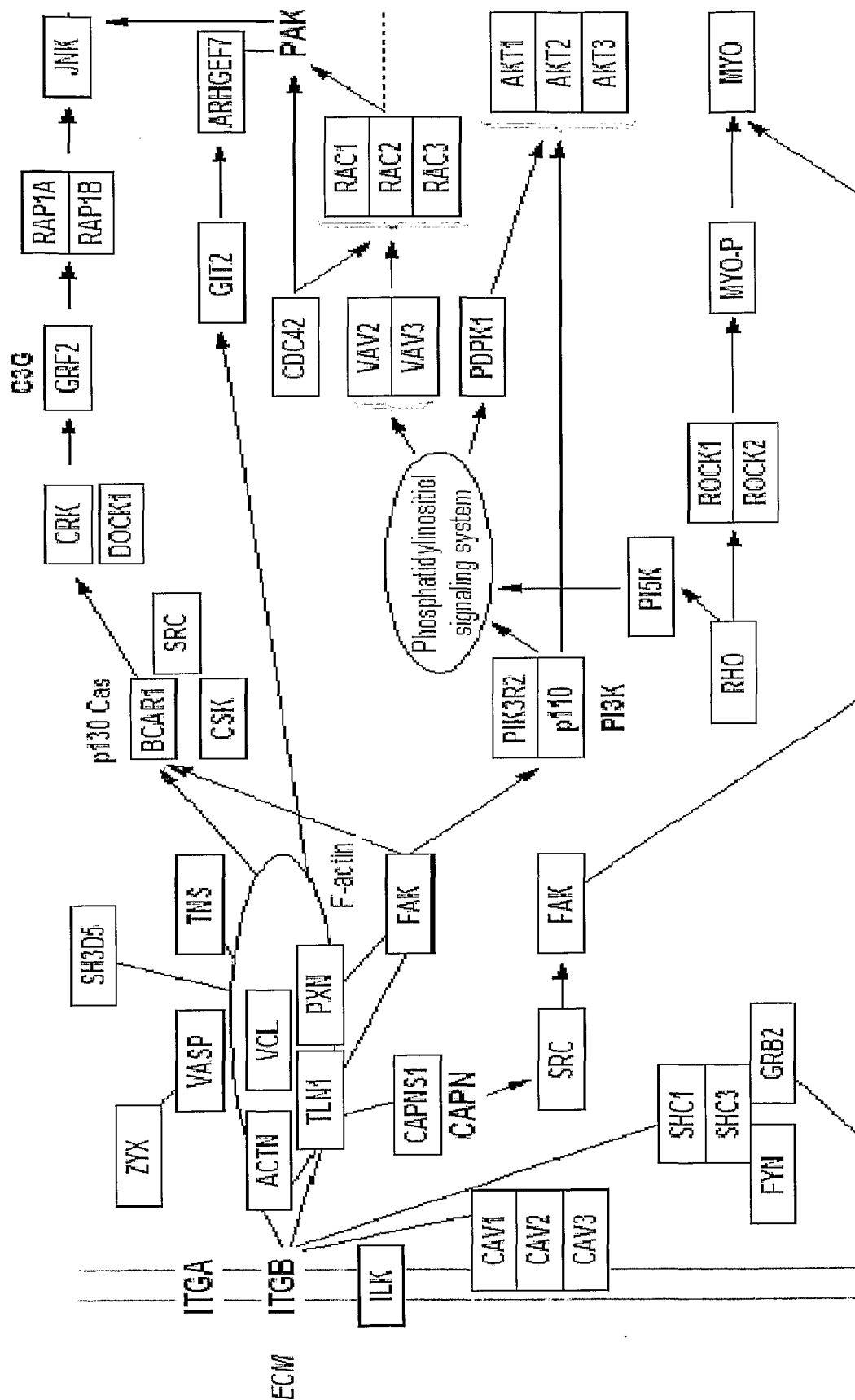


Figure 23 Pathway information

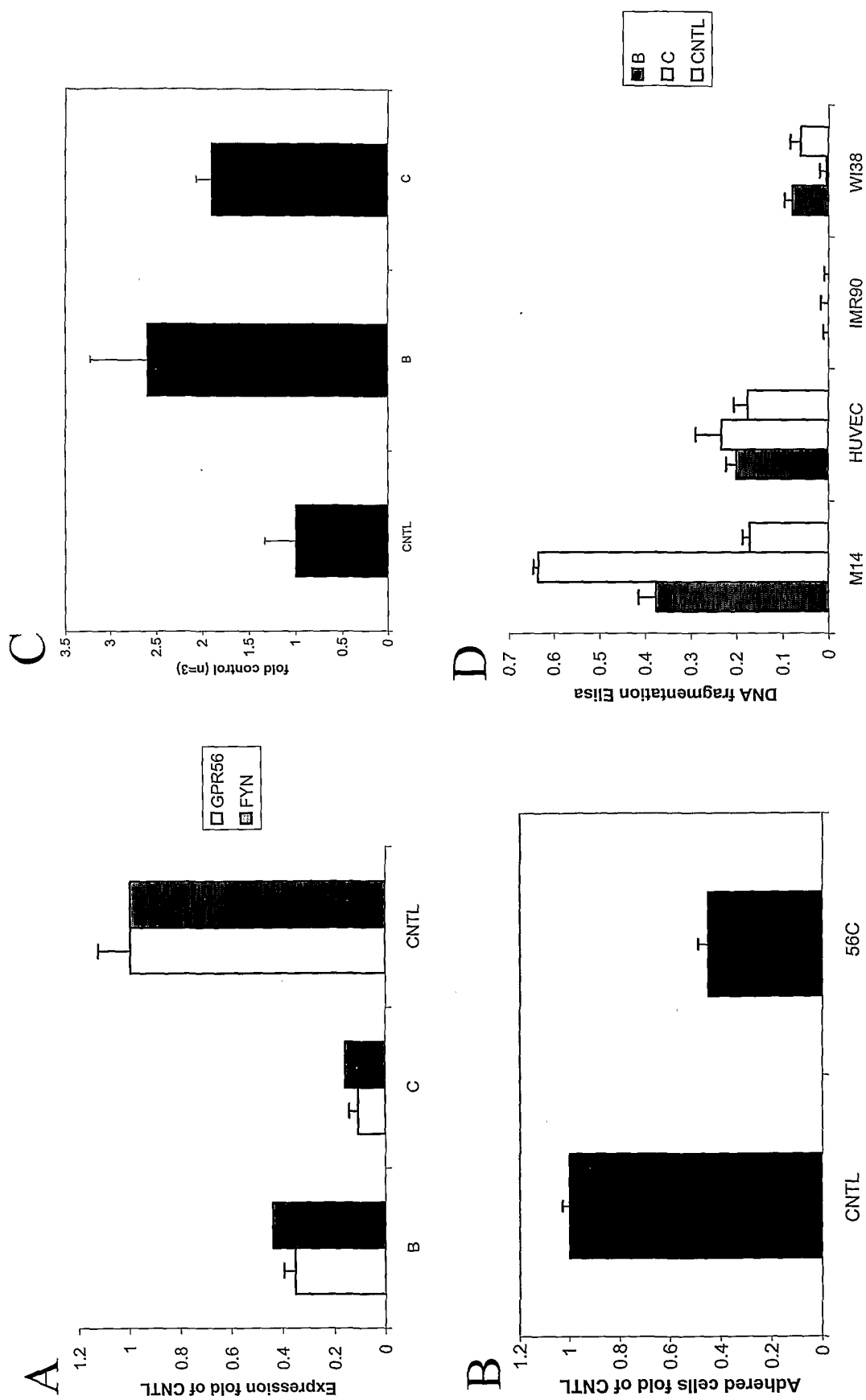


Figure 24 FYN (A), Adhesion (B), Anoikis (C), Elisa (D)

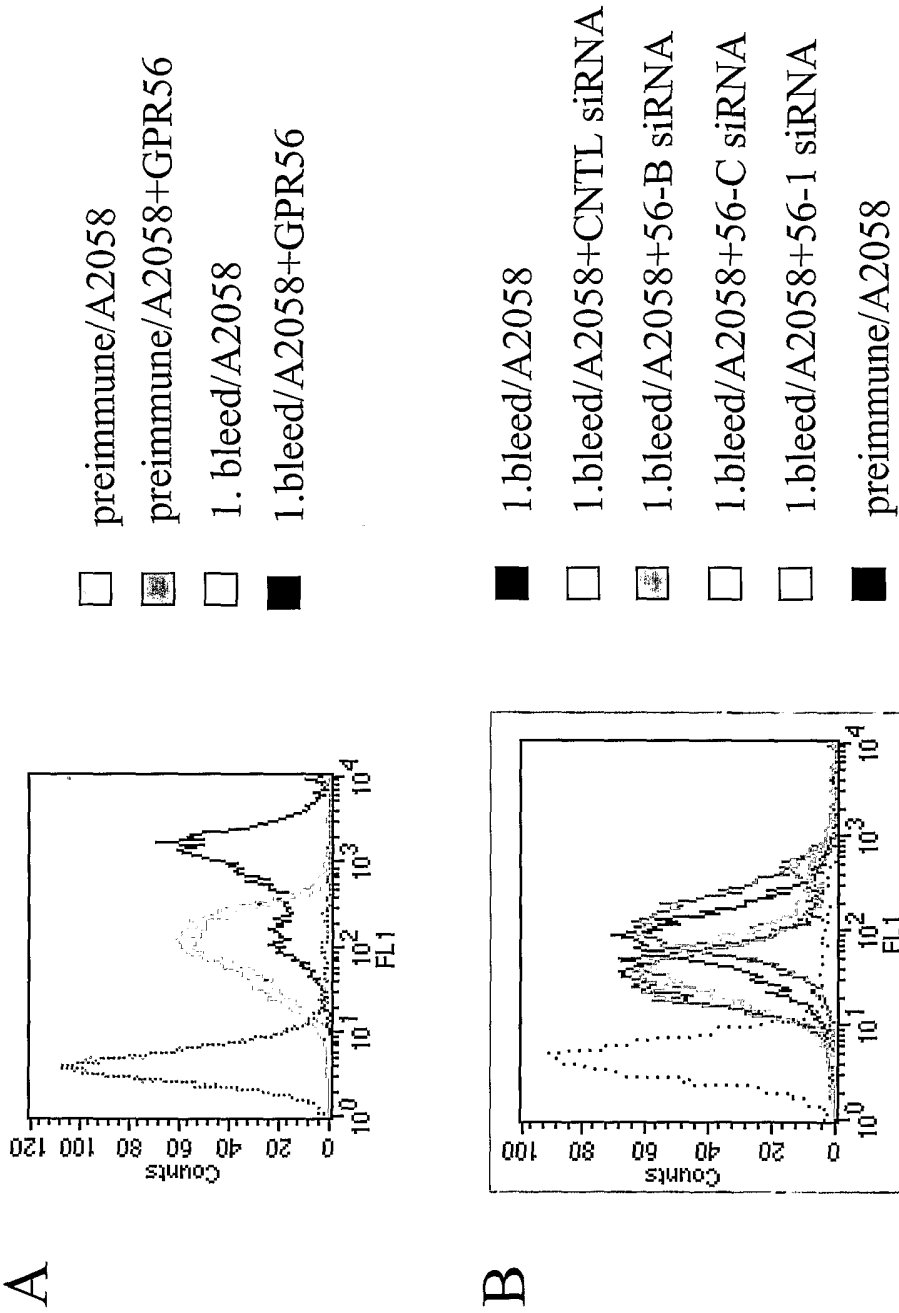


Figure 25

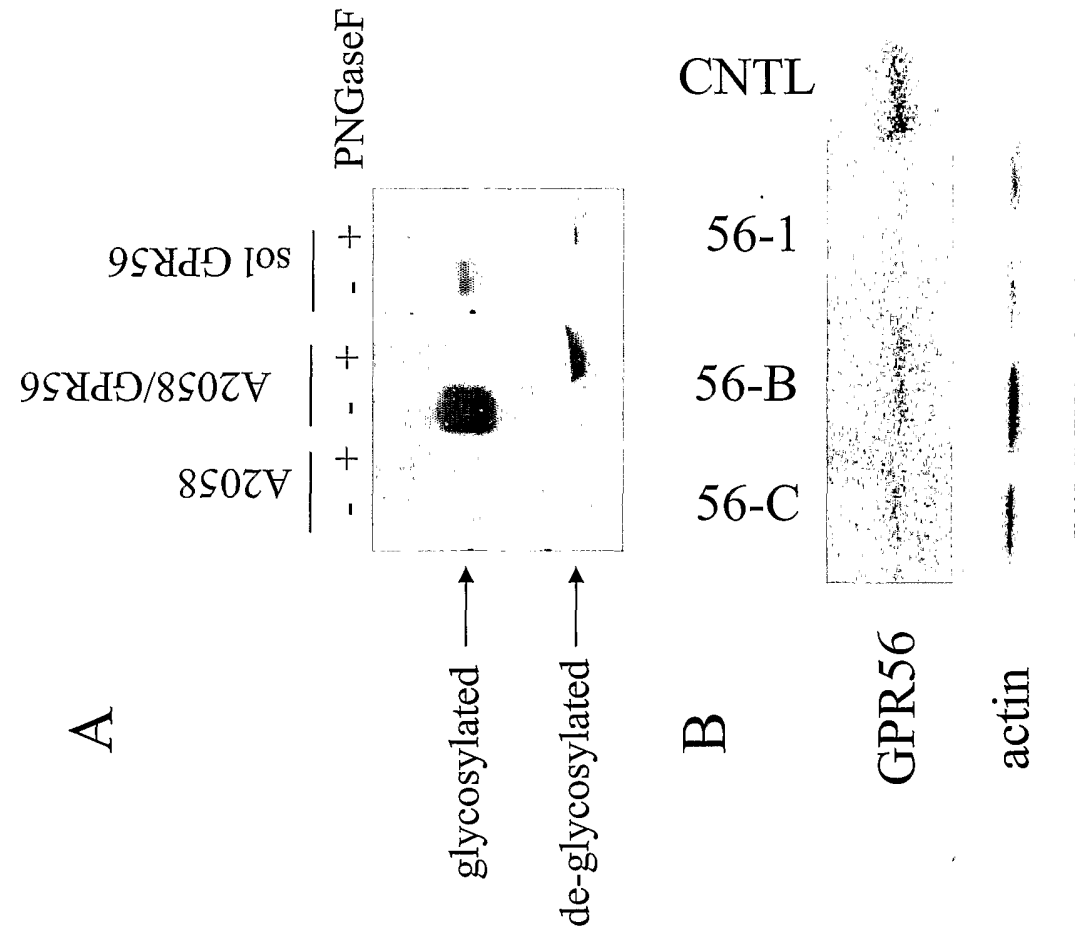


Figure 26

B



A



Figure 27

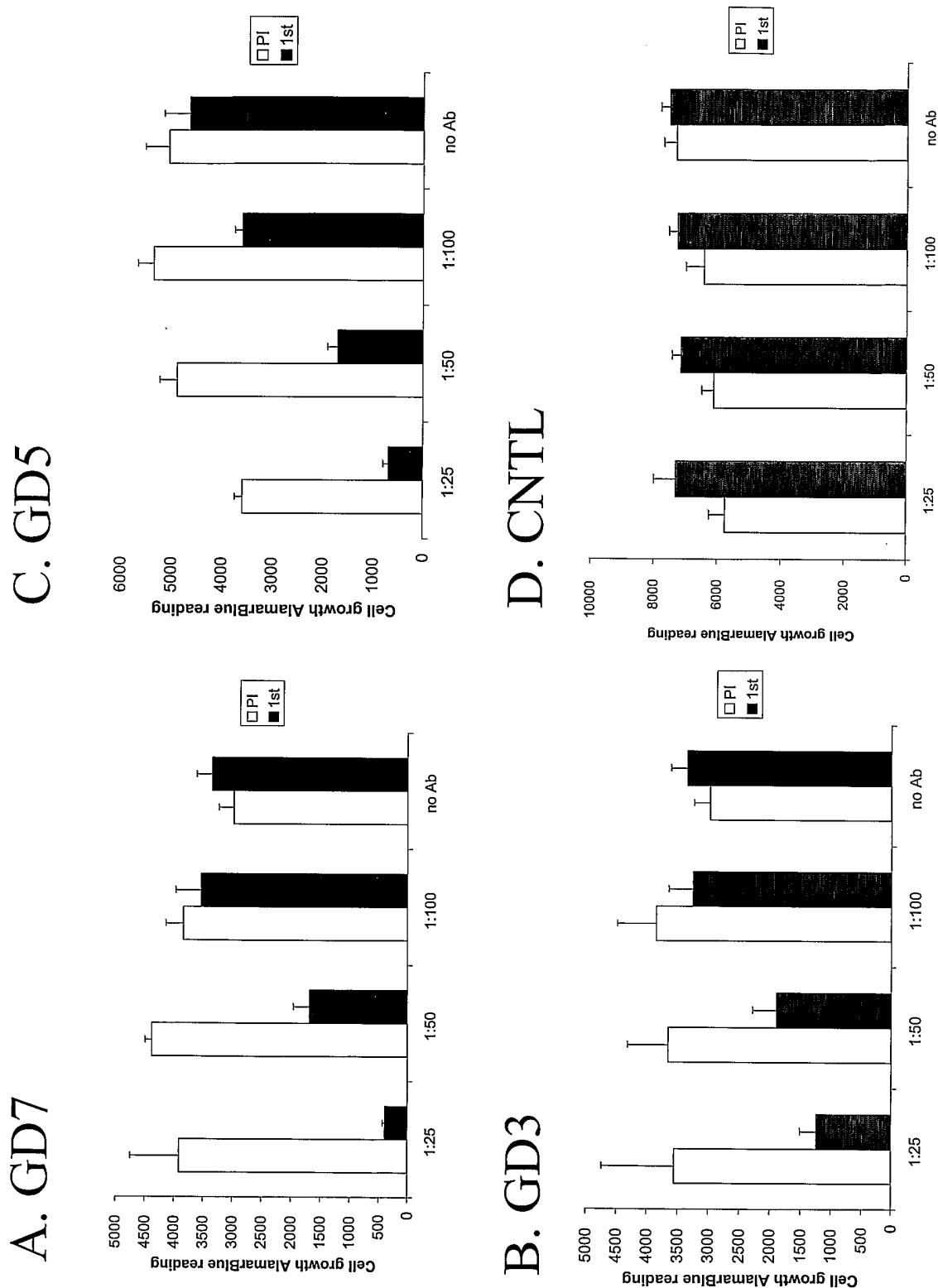


Figure 28

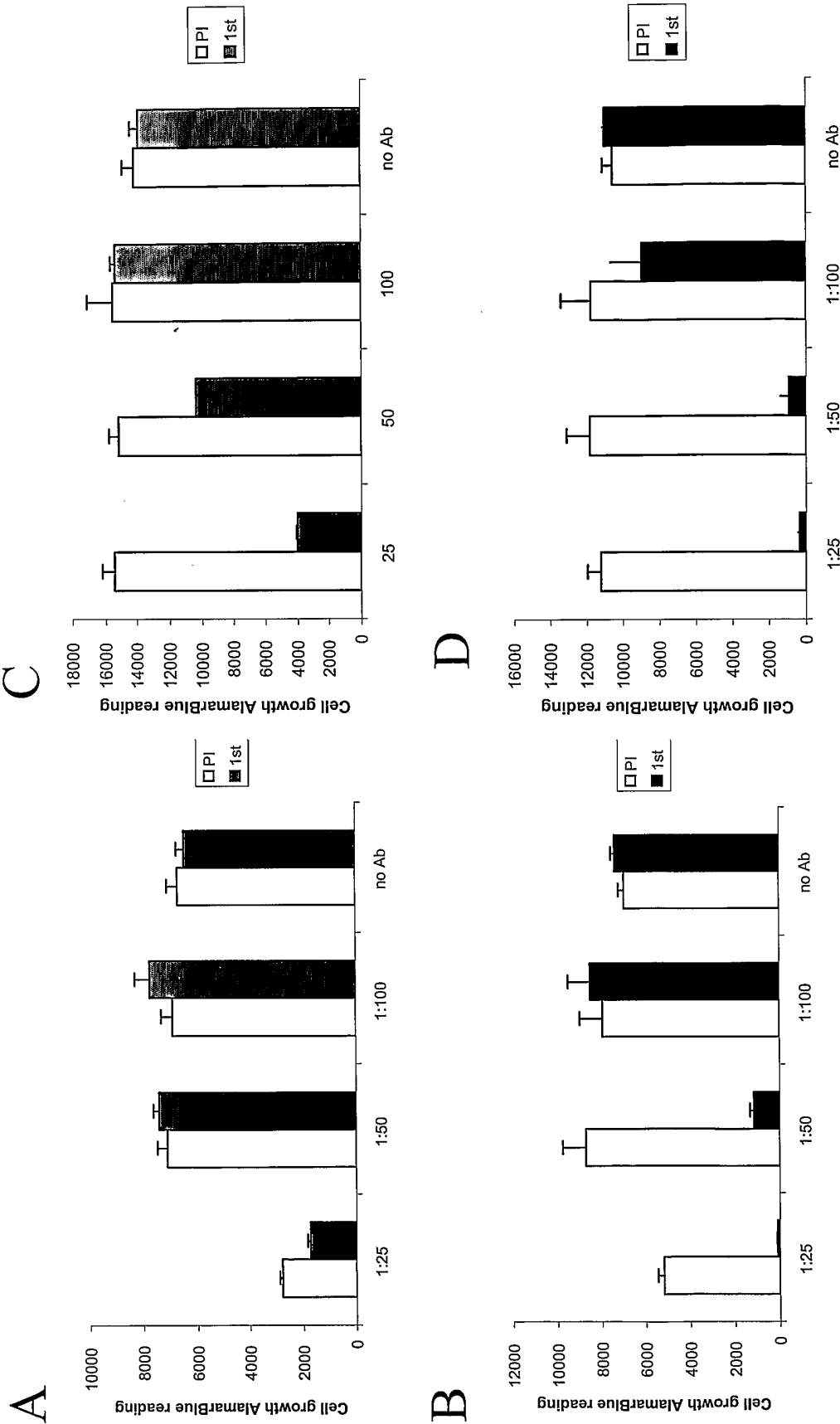


Figure 29 Cell pair in NIH3T3, A2058

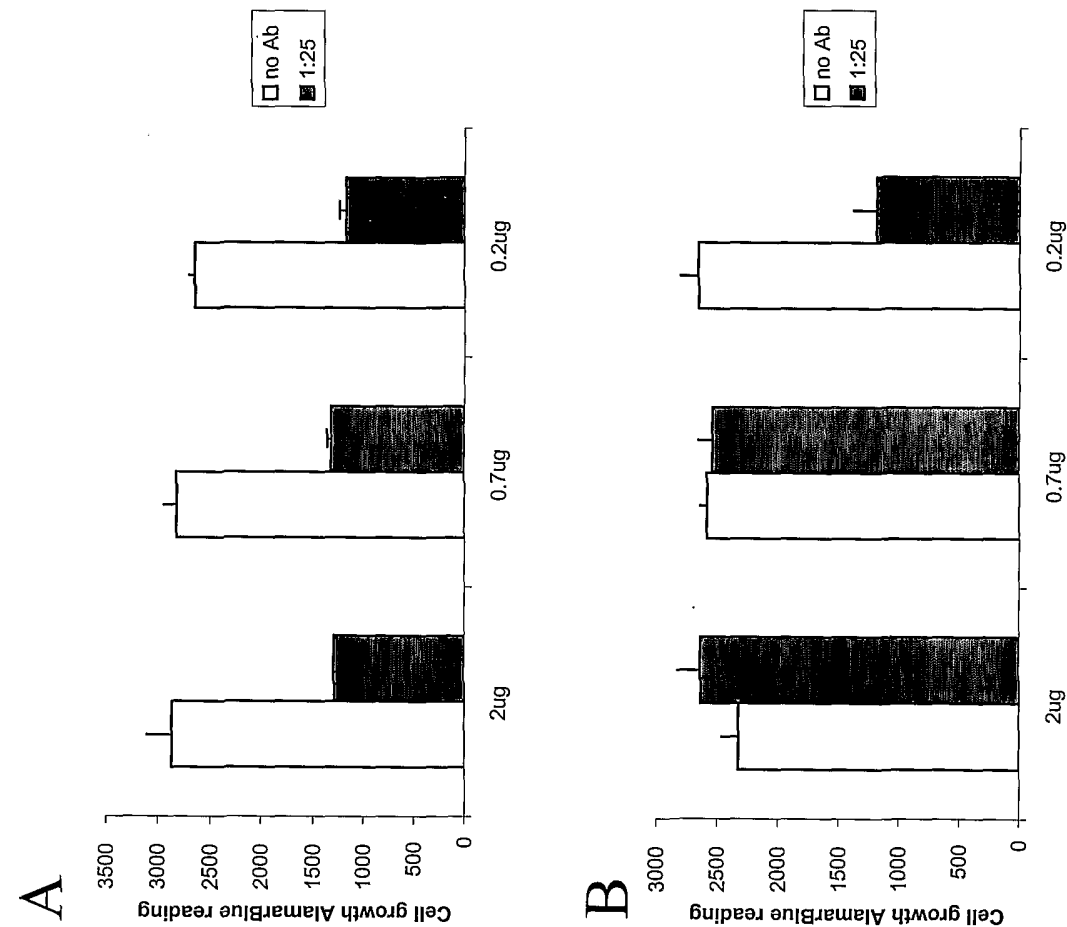


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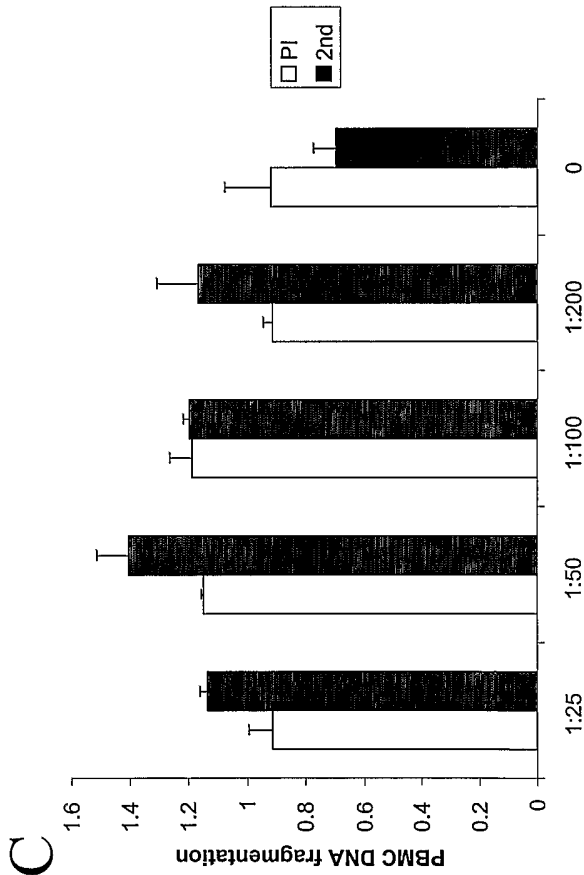
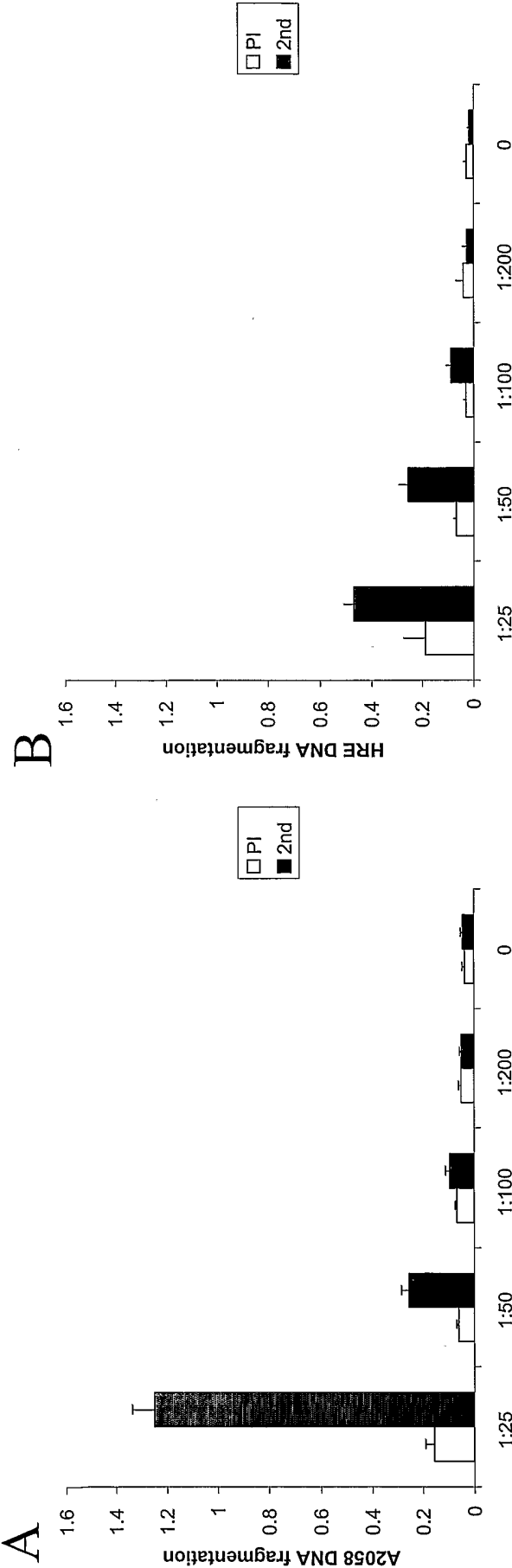


Figure 31

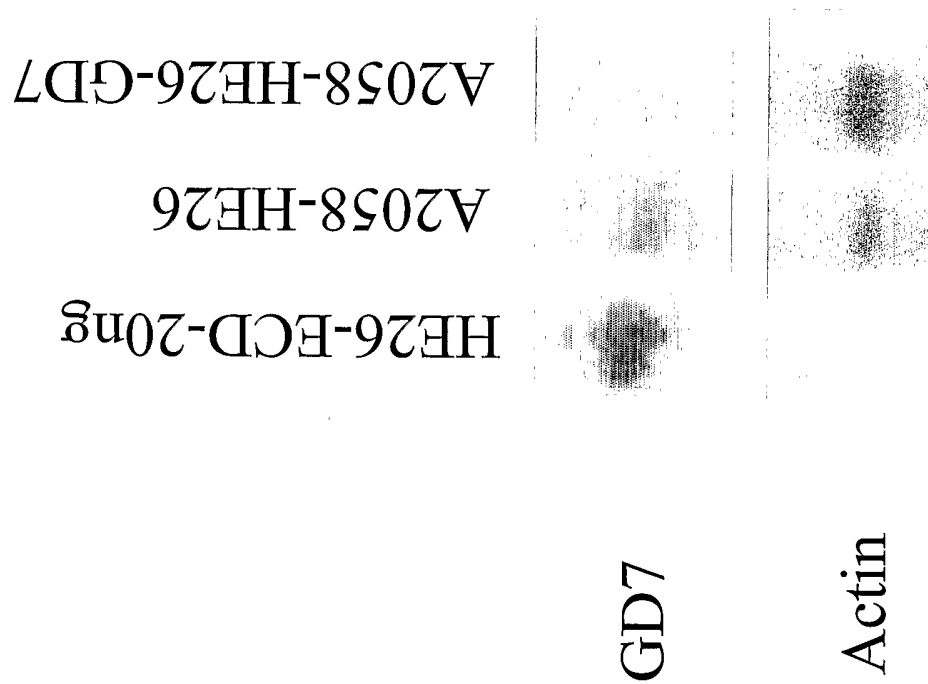


Figure 32

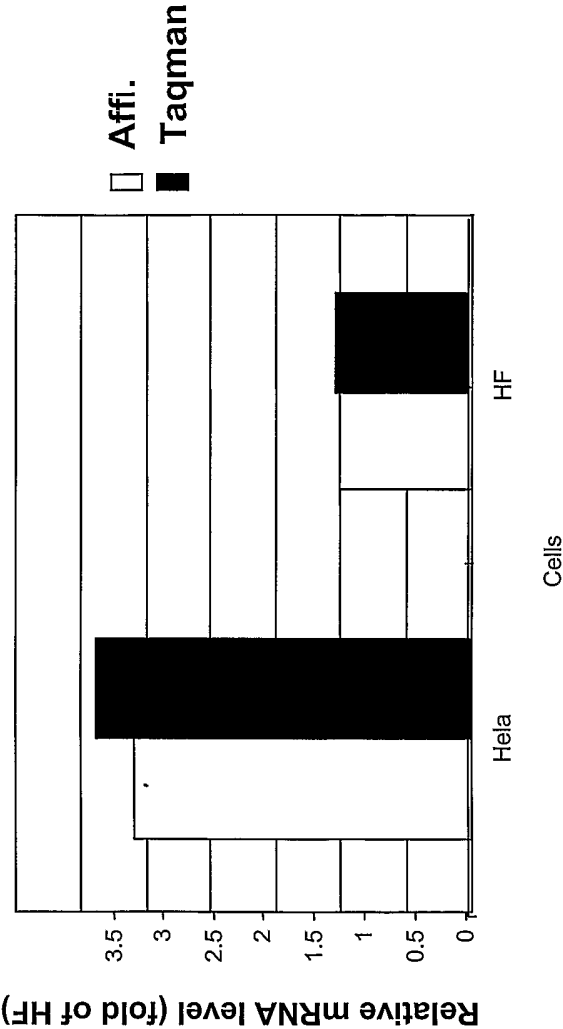


Figure 33

Stable knockdown HE60 in HOP62

Liquid culture

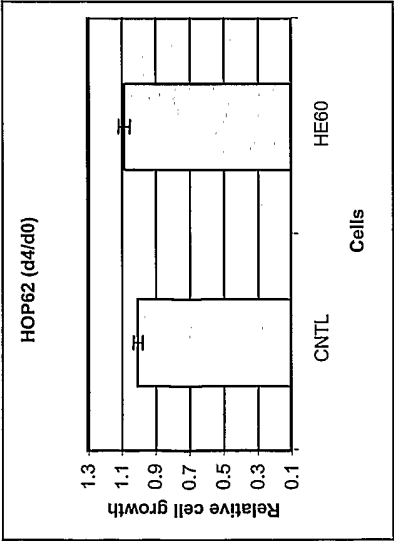


Figure 34A

Soft agar

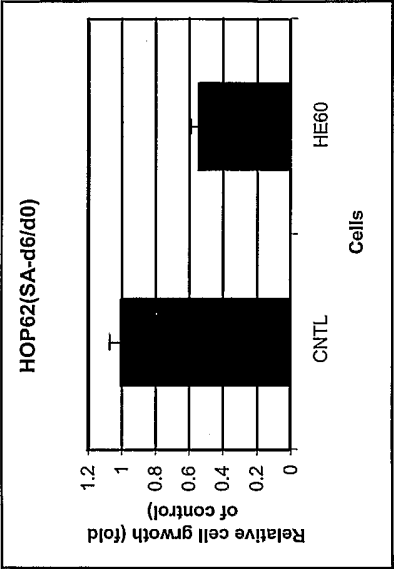


Figure 34B

Taqman

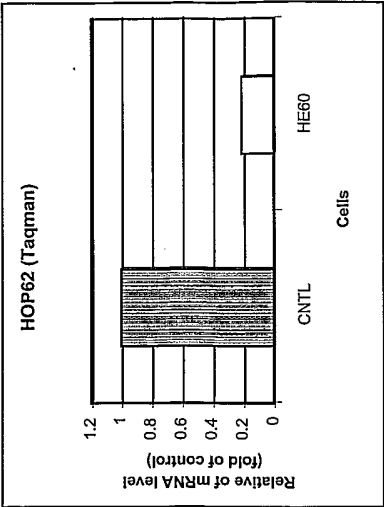
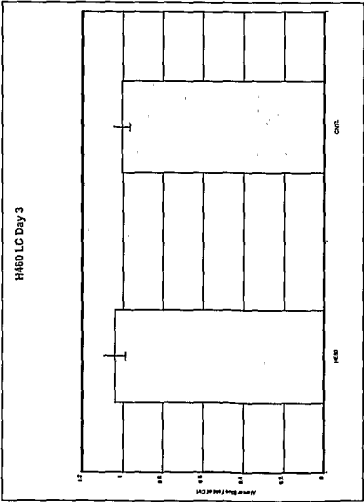


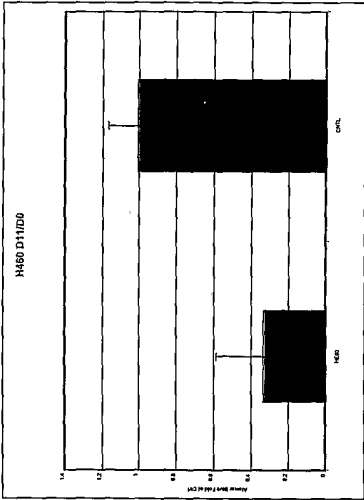
Figure 34C

Stable knockdown HE60 in H460

Liquid culture



Soft agar



Taqman

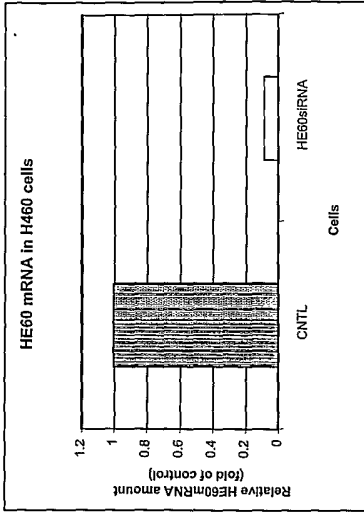


Figure 35A

Figure 35B

Figure 35C

Stable knockdown HE60 in A2058

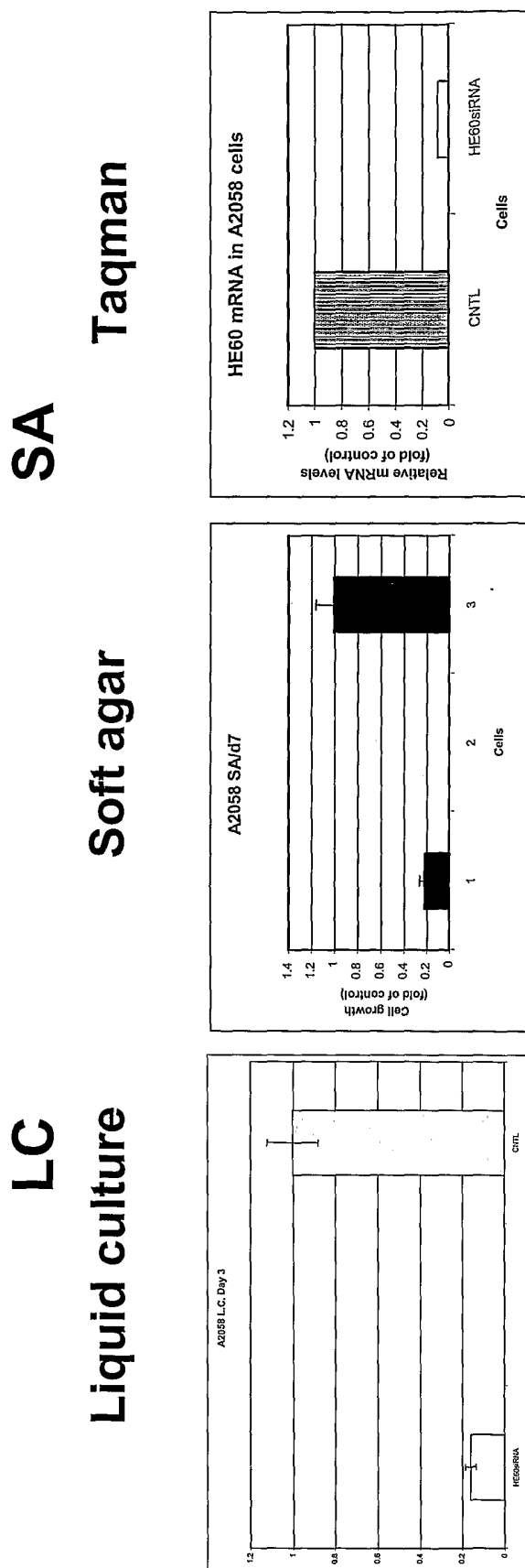


Figure 36A

Figure 36B

Figure 36C

Stable knockdown HE60 in PC3M/N

Liquid culture

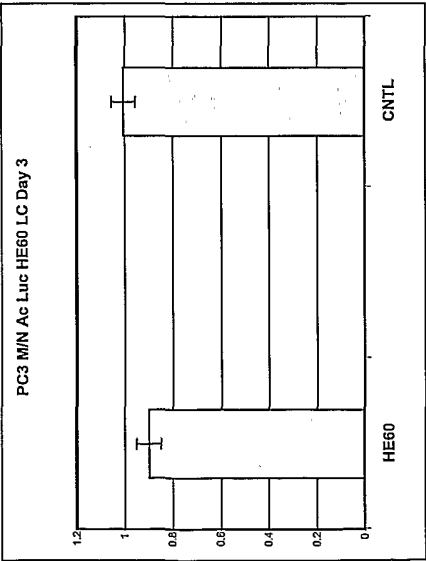


Figure 37A

Soft agar

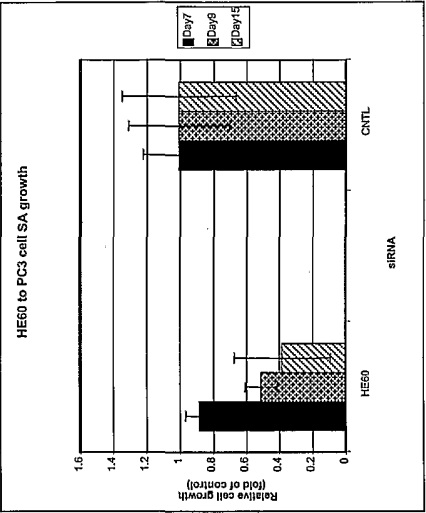


Figure 37B

Taqman

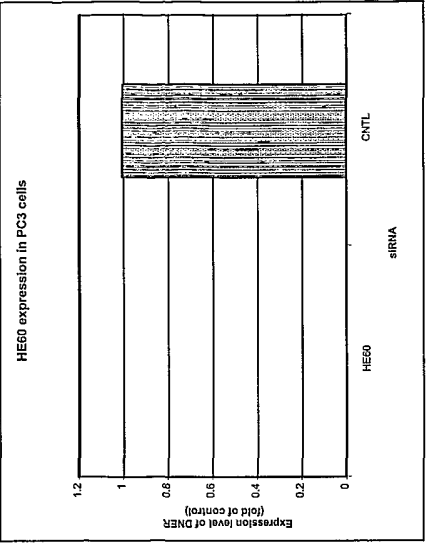


Figure 37C

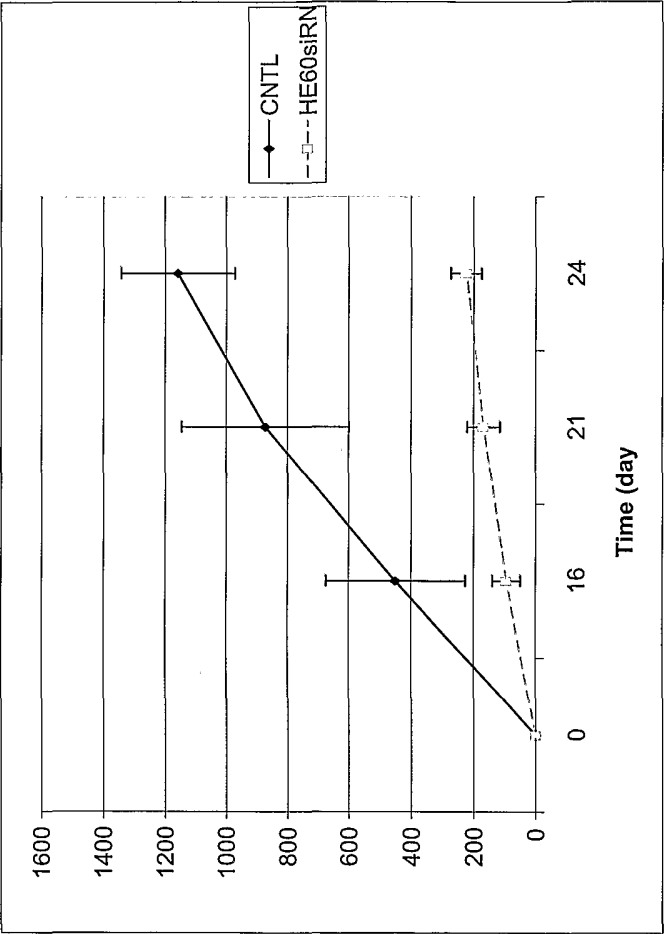


Figure 38

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```

ccccacaccc aaaagtgggc acatgtgctg acactgctgg gcctcagcct ggtccttggc 1860
ctgccctggg ccttgatctt cttctccttt gcttctggca ccttcagct tgcgtcctc 1920
taccttttca gcatcatcac ctccctccaa ggcttcctca tcttcactg gtactggtcc 1980
atgcggctgc agggccgggg tggccctcc cctctgaaga gcaactcaga cagcgccagg 2040
ctccccatca gctcgggcag cacctcgtcc agccgcatct ag 2082

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```

<210> 2
<211> 693
<212> PRT
<213> Homo sapiens

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<220>
<221> misc_feature
<222> (306)..(306)
<223> Can be His or Gln

```

```

<400> 2

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```

Met Thr Pro Gln Ser Leu Leu Gln Thr Thr Leu Phe Leu Leu Ser Leu
1           5           10           15

```

```

Leu Phe Leu Val Gln Gly Ala His Gly Arg Gly His Arg Glu Asp Phe
20           25           30

```

```

Arg Phe Cys Ser Gln Arg Asn Gln Thr His Arg Ser Ser Leu His Tyr
35           40           45

```

```

Lys Pro Thr Pro Asp Leu Arg Ile Ser Ile Glu Asn Ser Glu Glu Ala
50           55           60

```

```

Leu Thr Val His Ala Pro Phe Pro Ala Ala His Pro Ala Ser Arg Ser
65           70           75           80

```

```

Phe Pro Asp Pro Arg Gly Leu Tyr His Phe Cys Leu Tyr Trp Asn Arg
85           90           95

```

```

His Ala Gly Arg Leu His Leu Leu Tyr Gly Lys Arg Asp Phe Leu Leu
100          105          110

```

```

Ser Asp Lys Ala Ser Ser Leu Leu Cys Phe Gln His Gln Glu Glu Ser
115          120          125

```

```

Leu Ala Gln Gly Pro Pro Leu Leu Ala Thr Ser Val Thr Ser Trp Trp
130          135          140

```

```

Ser Pro Gln Asn Ile Ser Leu Pro Ser Ala Ala Ser Phe Thr Phe Ser
145          150          155          160

```

```

Phe His Ser Pro Pro His Thr Ala Ala His Asn Ala Ser Val Asp Met
165          170          175

```

```

Cys Glu Leu Lys Arg Asp Leu Gln Leu Leu Ser Gln Phe Leu Lys His
180          185          190

```

```

Pro Gln Lys Ala Ser Arg Arg Pro Ser Ala Ala Pro Ala Ser Gln Gln
195          200          205

```

Leu Gln Ser Leu Glu Ser Lys Leu Thr Ser Val Arg Phe Met Gly Asp
 210 215 220
 Met Val Ser Phe Glu Glu Asp Arg Ile Asn Ala Thr Val Trp Lys Leu
 225 230 235 240
 Gln Pro Thr Ala Gly Leu Gln Asp Leu His Ile His Ser Arg Gln Glu
 245 250 255
 Glu Glu Gln Ser Glu Ile Met Glu Tyr Ser Val Leu Leu Pro Arg Thr
 260 265 270
 Leu Phe Gln Arg Thr Lys Gly Arg Ser Gly Glu Ala Glu Lys Arg Leu
 275 280 285
 Leu Leu Val Asp Phe Ser Ser Gln Ala Leu Phe Gln Asp Lys Asn Ser
 290 295 300
 Ser Xaa Val Leu Gly Glu Lys Val Leu Gly Ile Val Val Gln Asn Thr
 305 310 315 320
 Lys Val Ala Asn Leu Thr Glu Pro Val Val Leu Thr Phe Gln His Gln
 325 330 335
 Leu Gln Pro Lys Asn Val Thr Leu Gln Cys Val Phe Trp Val Glu Asp
 340 345 350
 Pro Thr Leu Ser Ser Pro Gly His Trp Ser Ser Ala Gly Cys Glu Thr
 355 360 365
 Val Arg Arg Glu Thr Gln Thr Ser Cys Phe Cys Asn His Leu Thr Tyr
 370 375 380
 Phe Ala Val Leu Met Val Ser Ser Val Glu Val Asp Ala Val His Lys
 385 390 395 400
 His Tyr Leu Ser Leu Leu Ser Tyr Val Gly Cys Val Val Ser Ala Leu
 405 410 415
 Ala Cys Leu Val Thr Ile Ala Ala Tyr Leu Cys Ser Arg Val Pro Leu
 420 425 430
 Pro Cys Arg Arg Lys Pro Arg Asp Tyr Thr Ile Lys Val His Met Asn
 435 440 445
 Leu Leu Leu Ala Val Phe Leu Leu Asp Thr Ser Phe Leu Leu Ser Glu
 450 455 460
 Pro Val Ala Leu Thr Gly Ser Glu Ala Gly Cys Arg Ala Ser Ala Ile
 465 470 475 480
 Phe Leu His Phe Ser Leu Leu Thr Cys Leu Ser Trp Met Gly Leu Glu
 485 490 495
 Gly Tyr Asn Leu Tyr Arg Leu Val Val Glu Val Phe Gly Thr Tyr Val
 500 505 510
 Pro Gly Tyr Leu Leu Lys Leu Ser Ala Met Gly Trp Gly Phe Pro Ile

515 520 525
 Phe Leu Val Thr Leu Val Ala Leu Val Asp Val Asp Asn Tyr Gly Pro
 530 535 540
 Ile Ile Leu Ala Val His Arg Thr Pro Glu Gly Val Ile Tyr Pro Ser
 545 550 555 560
 Met Cys Trp Ile Arg Asp Ser Leu Val Ser Tyr Ile Thr Asn Leu Gly
 565 570 575
 Leu Phe Ser Leu Val Phe Leu Phe Asn Met Ala Met Leu Ala Thr Met
 580 585 590
 Val Val Gln Ile Leu Arg Leu Arg Pro His Thr Gln Lys Trp Ser His
 595 600 605
 Val Leu Thr Leu Leu Gly Leu Ser Leu Val Leu Gly Leu Pro Trp Ala
 610 615 620
 Leu Ile Phe Phe Ser Phe Ala Ser Gly Thr Phe Gln Leu Val Val Leu
 625 630 635 640
 Tyr Leu Phe Ser Ile Ile Thr Ser Phe Gln Gly Phe Leu Ile Phe Ile
 645 650 655
 Trp Tyr Trp Ser Met Arg Leu Gln Ala Arg Gly Gly Pro Ser Pro Leu
 660 665 670
 Lys Ser Asn Ser Asp Ser Ala Arg Leu Pro Ile Ser Ser Gly Ser Thr
 675 680 685
 Ser Ser Ser Arg Ile
 690

<210> 3
 <211> 2214
 <212> DNA
 <213> Homo sapiens

<400> 3
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 ctgctgctgc tcggagcggg gccccgaggc agctccctgg ccaaccgggt gcccgccgag 120
 cccttgtctg cgcgggggcc gtgcgcgcgc cagccctgcc ggaatggggg tgtgtgcacc 180
 tcgcgccttg agccggaccc gcagcacccc gccccgcgcg gcgagccttg ctacagctgc 240
 acctgccccg ccgggatctc cggcgccaac tgccagcttg ttgcagatcc ttgtgccagc 300
 aacccttgct accatggcaa ctgcagcagc agcagcagca gcagcagcga tggctacctc 360
 tgcatttgca atgaaggcta tgaaggtoac aactgtgaac aggcacttcc cagtctccca 420
 gccactggct ggaccgaatc catggcacc cgcagcttc agcctgttcc tgctactcag 480
 gagcctgaca aaatcctgcc tcgtctcag gcaacgggtga cactgcctac ctggcagccg 540
 aaaacagggc agaaagtgt agaaatgaaa tgggatcaag tggaggtgat ccagatatt 600
 gcctgtggga atgccagttc taacagctct gcgggtggcc gcctggatc ctttgaagtg 660
 ccacagaaca cctcagtcaa gattcggcaa gatgccactg cctcactgat tttgctctgg 720

aaggtcacgg ccacaggatt ccaacagtgc tccctcatag atggacgaag tgtgaccccc 780
cttcaggctt cagggggact ggtcctcctg gaggagatgc tcgccttggg gaataatcac 840
tttattgggt ttgtgaatga ttctgtgact aagtctattg tggctttgcg cttaactctg 900
gtggtgaagg tcagcacctg tgtgccgggg gagagtcacg caaatgactt ggagtgttca 960
ggaaaaggaa aatgcaccac gaagccgtca gaggcaactt tttcctgtac ctgtgaggag 1020
cagtacgtgg gtactttctg tgaagaatac gatgcttgcc agaggaaacc ttgccaaaac 1080
aacgcgagct gtattgatgc aaatgaaaag caagatggga gcaatttcac ctgtgtttgc 1140
cttcctggtt atactggaga gctttgccag tccaagattg attactgcat cctagaccca 1200
tgcagaaatg gagcaacatg catttccagt ctcaagtggat tcacctgcca gtgtccagaa 1260
ggatacttcg gatctgcttg tgaagaaaag gtggacctct gcgcctcgtc tccgtgccag 1320
aacaacggca cctgctatgt ggacggggta cactttacct gcaactgcag cccgggcttc 1380
acagggccga cctgtgccca gcttattgac ttctgtgccc tcagccctg tgctcatggc 1440
acgtgccga gcgtgggcac cagctacaaa tgcctctgtg atccaggtta coatggcctc 1500
tactgtgagg aggaatataa tgagtgcctc tccgtccat gcctgaatgc agccacctgc 1560
agggacctcg ttaatggcta tgagtgtgtg tgcctggcag aatacaaagg aacacactgt 1620
gaattgtaca aggatccctg cgctaacgtc agctgtctga acggagccac ctgtgacagc 1680
gacggcctga atggcacgtg catctgtgca cccgggttta caggtgaaga gtgcgacatt 1740
gacataaatg aatgtgacag taaccctgc caccatggtg ggagctgcct ggaccagccc 1800
aatggttata actgccactg ccgcgatggt tgggtgggag caaactgtga gatccacctc 1860
caatggaagt ccgggcacat ggcgagagc ctaccaaca tgccacggca ctccctctac 1920
atcatcattg gagccctctg cgtggccttc atccttatgc tgatcatcct gatcgtgggg 1980
atttgccgca tcagccgcat tgaataccag ggttcttcca ggccagccta tgaggagttc 2040
tacaactgcc gcagcatcga cagcgagttc agcaatgcca ttgcatccat ccggcatgcc 2100
aggtttggaa agaaatcccg gcctgcaatg tatgatgtga gcccacgc ctatgaagat 2160
tacagtcctg atgacaaacc cttggtcaca ctgattaaaa ctaaagattt gtaa 2214

<210> 4
<211> 737
<212> PRT
<213> Homo sapiens

<400> 4

Met Gln Pro Arg Arg Ala Gln Ala Pro Gly Ala Gln Leu Leu Pro Ala
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Leu Ala Leu Leu Leu Leu Leu Gly Ala Gly Pro Arg Gly Ser Ser
20 25 30

Leu Ala Asn Pro Val Pro Ala Ala Pro Leu Ser Ala Pro Gly Pro Cys
35 40 45

Ala Ala Gln Pro Cys Arg Asn Gly Gly Val Cys Thr Ser Arg Pro Glu
50 55 60

Pro Asp Pro Gln His Pro Ala Pro Ala Gly Glu Pro Gly Tyr Ser Cys
65 70 75 80

Thr Cys Pro Ala Gly Ile Ser Gly Ala Asn Cys Gln Leu Val Ala Asp
 85 90 95
 Pro Cys Ala Ser Asn Pro Cys His His Gly Asn Cys Ser Ser Ser Ser
 100 105 110
 Ser Ser Ser Ser Asp Gly Tyr Leu Cys Ile Cys Asn Glu Gly Tyr Glu
 115 120 125
 Gly Pro Asn Cys Glu Gln Ala Leu Pro Ser Leu Pro Ala Thr Gly Trp
 130 135 140
 Thr Glu Ser Met Ala Pro Arg Gln Leu Gln Pro Val Pro Ala Thr Gln
 145 150 155 160
 Glu Pro Asp Lys Ile Leu Pro Arg Ser Gln Ala Thr Val Thr Leu Pro
 165 170 175
 Thr Trp Gln Pro Lys Thr Gly Gln Lys Val Val Glu Met Lys Trp Asp
 180 185 190
 Gln Val Glu Val Ile Pro Asp Ile Ala Cys Gly Asn Ala Ser Ser Asn
 195 200 205
 Ser Ser Ala Gly Gly Arg Leu Val Ser Phe Glu Val Pro Gln Asn Thr
 210 215 220
 Ser Val Lys Ile Arg Gln Asp Ala Thr Ala Ser Leu Ile Leu Leu Trp
 225 230 235 240
 Lys Val Thr Ala Thr Gly Phe Gln Gln Cys Ser Leu Ile Asp Gly Arg
 245 250 255
 Ser Val Thr Pro Leu Gln Ala Ser Gly Gly Leu Val Leu Leu Glu Glu
 260 265 270
 Met Leu Ala Leu Gly Asn Asn His Phe Ile Gly Phe Val Asn Asp Ser
 275 280 285
 Val Thr Lys Ser Ile Val Ala Leu Arg Leu Thr Leu Val Val Lys Val
 290 295 300
 Ser Thr Cys Val Pro Gly Glu Ser His Ala Asn Asp Leu Glu Cys Ser
 305 310 315 320
 Gly Lys Gly Lys Cys Thr Thr Lys Pro Ser Glu Ala Thr Phe Ser Cys
 325 330 335
 Thr Cys Glu Glu Gln Tyr Val Gly Thr Phe Cys Glu Glu Tyr Asp Ala
 340 345 350
 Cys Gln Arg Lys Pro Cys Gln Asn Asn Ala Ser Cys Ile Asp Ala Asn
 355 360 365
 Glu Lys Gln Asp Gly Ser Asn Phe Thr Cys Val Cys Leu Pro Gly Tyr
 370 375 380

Thr Gly Glu Leu Cys Gln Ser Lys Ile Asp Tyr Cys Ile Leu Asp Pro
 385 390 395 400
 Cys Arg Asn Gly Ala Thr Cys Ile Ser Ser Leu Ser Gly Phe Thr Cys
 405 410 415
 Gln Cys Pro Glu Gly Tyr Phe Gly Ser Ala Cys Glu Glu Lys Val Asp
 420 425 430
 Leu Cys Ala Ser Ser Pro Cys Gln Asn Asn Gly Thr Cys Tyr Val Asp
 435 440 445
 Gly Val His Phe Thr Cys Asn Cys Ser Pro Gly Phe Thr Gly Pro Thr
 450 455 460
 Cys Ala Gln Leu Ile Asp Phe Cys Ala Leu Ser Pro Cys Ala His Gly
 465 470 475 480
 Thr Cys Arg Ser Val Gly Thr Ser Tyr Lys Cys Leu Cys Asp Pro Gly
 485 490 495
 Tyr His Gly Leu Tyr Cys Glu Glu Glu Tyr Asn Glu Cys Leu Ser Ala
 500 505 510
 Pro Cys Leu Asn Ala Ala Thr Cys Arg Asp Leu Val Asn Gly Tyr Glu
 515 520 525
 Cys Val Cys Leu Ala Glu Tyr Lys Gly Thr His Cys Glu Leu Tyr Lys
 530 535 540
 Asp Pro Cys Ala Asn Val Ser Cys Leu Asn Gly Ala Thr Cys Asp Ser
 545 550 555 560
 Asp Gly Leu Asn Gly Thr Cys Ile Cys Ala Pro Gly Phe Thr Gly Glu
 565 570 575
 Glu Cys Asp Ile Asp Ile Asn Glu Cys Asp Ser Asn Pro Cys His His
 580 585 590
 Gly Gly Ser Cys Leu Asp Gln Pro Asn Gly Tyr Asn Cys His Cys Pro
 595 600 605
 His Gly Trp Val Gly Ala Asn Cys Glu Ile His Leu Gln Trp Lys Ser
 610 615 620
 Gly His Met Ala Glu Ser Leu Thr Asn Met Pro Arg His Ser Leu Tyr
 625 630 635 640
 Ile Ile Ile Gly Ala Leu Cys Val Ala Phe Ile Leu Met Leu Ile Ile
 645 650 655
 Leu Ile Val Gly Ile Cys Arg Ile Ser Arg Ile Glu Tyr Gln Gly Ser
 660 665 670
 Ser Arg Pro Ala Tyr Glu Glu Phe Tyr Asn Cys Arg Ser Ile Asp Ser
 675 680 685
 Glu Phe Ser Asn Ala Ile Ala Ser Ile Arg His Ala Arg Phe Gly Lys

690

695

700

Lys Ser Arg Pro Ala Met Tyr Asp Val Ser Pro Ile Ala Tyr Glu Asp
 705 710 715 720

Tyr Ser Pro Asp Asp Lys Pro Leu Val Thr Leu Ile Lys Thr Lys Asp
 725 730 735

Leu

<210> 5
 <211> 23
 <212> DNA
 <213> Artificial sequence

<220>
 <223> SiRNA target sequence

<400> 5
 gaaggtgcac atgaacctgc tgc 23

<210> 6
 <211> 23
 <212> DNA
 <213> Artificial sequence

<220>
 <223> SiRNA target sequence

<400> 6
 gaaatgtggc tccagttgct etc 23

<210> 7
 <211> 93
 <212> DNA
 <213> Artificial sequence

<220>
 <223> Single strand DNA oligonucleotide

<400> 7
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 tgtgcacctt caaacaaggc ttttctcaa ggg 93

<210> 8
 <211> 93
 <212> DNA
 <213> Artificial sequence

<220>
 <223> Single strand DNA oligonucleotide

<400> 8
 tgctggatcc aaaaaagaaa tgtggctcca gttgctctct ctcttgaaga gagcaactgg 60
 agccacattt caaacaaggc ttttctcaa ggg 93

<210> 9
 <211> 20
 <212> DNA
 <213> Artificial sequence

<220>
 <223> Single strand DNA oligonucleotide

<400> 9
 gaactagtgg atccgacgcc 20

<210> 10
<211> 21
<212> RNA
<213> Artificial sequence

<220>
<223> SiRNA oligonucleotide

<400> 10
cgucagcugu cugaacggau u 21

<210> 11
<211> 21
<212> RNA
<213> Artificial sequence

<220>
<223> SiRNA oligonucleotide

<400> 11
uccguucaga cagcugacgu u 21

<210> 12
<211> 21
<212> RNA
<213> Artificial sequence

<220>
<223> SiRNA oligonucleotide

<400> 12
ggcuauagaag gucccaacuu u 21

<210> 13
<211> 21
<212> RNA
<213> Artificial sequence

<220>
<223> SiRNA oligonucleotide

<400> 13
aguugggacc uucauagccu u 21