(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 May 2009 (14.05.2009)

PCT

(10) International Publication Number
WO 2009/062104 A2

(51) International Patent Classification:
A61K 48/00 (2006.01)

(21) International Application Number:
PCT/US2008/082892

(22) International Filing Date:
7 November 2008 (07.11.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/986,716 9 November 2007 (09.11.2007) US

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

[Continued on next page]

(54) **Title:** ABERRANT EXPRESSION OF ZINC TRANSPORTER ZIP4 (SLC39A4) SIGNIFICANTLY CONTRIBUTES TO HUMAN PANCREATIC CANCER PATHOGENESIS AND PROGRESSION

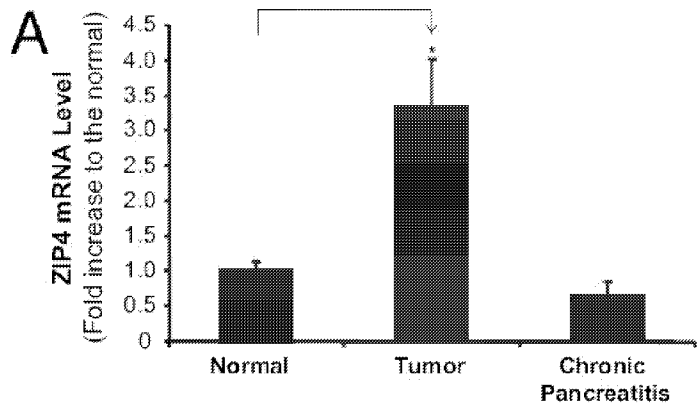

FIG. 1A

(57) **Abstract:** This invention identifies a previously uncharacterized factor in pancreatic malignancy, ZIP4, and thereby identifies a target for cancer therapy. Agents that inhibit ZIP4 provide methods and compositions to treat cancer, specifically, pancreatic cancer.

WO 2009/062104 A2

— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

Aberrant Expression of Zinc Transporter ZIP4 (SLC39A4) Significantly Contributes to Human Pancreatic Cancer Pathogenesis and Progression

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application 60/986,716 filed November 9, 2007, which is incorporated by reference herein in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] The present invention was developed at least in part with funds from National Institutes of Health Grants DK31127 and R37 GM55425. The United States Government has certain rights in the invention.

TECHNICAL FIELD

[0003] The technical field of the invention is cancer therapy and modulation of zinc transport mechanisms.

BACKGROUND OF THE INVENTION

[0004] Pancreatic cancer (PC) is the fourth leading cause of cancer-related death in North America. Although some progress has been made in surgery, chemotherapy, and radiation therapy in recent decades, the incidence of PC still remains equal to the mortality rate (Landis *et al.*, 1998; Torrisani and Buscail, 2002; Warshaw and Fernandez-del Castillo, 1992). In the United States, approximately 37,000 patients are diagnosed with PC every year, and all will die of this malignancy. The poor survival statistics are due to the fact that there are no reliable tests for early diagnosis and no effective therapies once the metastasis has occurred. Eighty percent of pancreatic adenocarcinomas are unresectable in the patients with clinical symptoms (Warshaw and Fernandez-del Castillo, 1992; Fisher and Berger, 2003). Although some progress has been made in surgery, chemotherapy, and radiation therapy in recent decades, the incidence of pancreatic cancer still remains equal to the mortality rate (Landis *et al.*, 1998; Torrisani and Buscail, 2002; Warshaw and Fernandez-del, 1992). Even the minority of patients who undergo resection will develop local recurrence and die of this disease within 15 months of diagnosis. Clearly, there is a need to understand more about the molecular mechanisms of PC pathogenesis and to develop effective treatment for PC. Important insights have been gained by studying the

progressive development of precursor lesions and invasive cancers in animal models of PC. Genomic profile studies, such as serial analysis of gene expression (SAGE), microarray analysis, and DNA sequencing have also been used to identify genes over-expressed in PC and to detect genetic mutations in oncogenes and tumor suppressor genes (Day *et al.*, 1996; Hruban *et al.*, 1993; DiGiuseppe *et al.*, 1994; Sturm *et al.*, 1998; van Heek *et al.*, 2002). The identification of these over-expressed genes might lead to development of new markers and screening techniques as well as new gene therapy strategies. However, many of these genes are non-specific in PC, which is highly heterogeneous. Therefore, it must be beneficial for diagnosis and therapeutics of PC to target genes that are either more specific in PC, or genes that are involved in multiple pathways such as survival, metabolism, and nutrition uptake. Surface ion transporters, transcriptional factors, and cytokines might be good candidates for the above reasons. Ion transporters provide important ions for many biological processes, and are essential for the activities of many transcription factors and enzymes, which play important roles in cancer pathogenesis. Signal transducer and activator of transcription 3 (STAT3) is an important transcription factor and constitutively activated in the majority of PC, and it has been shown to be linked with many other pathways and play a central role in PC pathogenesis. Cytokines are also important in PC progression and interact with many signaling pathways in PC cells. PC is a malignancy characterized by secreting multiple proinflammatory cytokines such as interleukin-6 (IL-6), interleukin-8 (IL-8), and other Th2 type cytokines, which promotes the progression of PC by providing a more favorable microenvironment for tumor growth (Feurino *et al.*, 2006; Feurino *et al.*, 2007; Xie, 2001; Xiao *et al.*, 2004). Despite the implication in many malignant cells, the roles of altered cellular metabolism or the interaction between genetics and the environment as an essential factor in cancers, especially in pancreatic cancer, has been largely ignored (Costello and Franklin, 2006; Quraishi *et al.*, 2005).

[0005] Zinc is an essential trace element and catalytic/structural component used by many metalloenzymes and transcription factors that contain zinc-finger motifs (Mao *et al.*, 2007; Wang *et al.*, 2002). Zinc deficiency in animals leads to growth retardation, decreased food intake, impaired DNA synthesis, immune system dysfunction, and severe dermatitis (King and Cousins, 2005). Zinc availability is also important for tumor growth and progression, because zinc is a critical component for many enzymes, such as carbonic anhydrase and matrix metalloproteinases (MMPs), which are involved in hypoxia, angiogenesis, cell proliferation, and metastasis of cancer (Juhasz *et al.*, 2003; Garcea *et al.*, 2006). High zinc concentrations are toxic

to the cells, therefore, cells have evolved a complex system to maintain the balance of zinc uptake, intracellular storage, and efflux (Kim *et al.*, 2004; Liuzzi and Cousins, 2004). Two solute-linked carrier (SLC) gene families were identified in zinc transport, SLC30, which encodes for zinc transporter (ZnT) proteins, and SLC39, which encodes for Zrt-, Irt-like proteins (ZIP) (Liuzzi and Cousins, 2004; Guerinot, 2000; Eide, 2004). They appear to have opposite roles in cellular zinc homeostasis. ZnT transporters reduce intracellular zinc availability by promoting zinc efflux from cells or into intracellular vesicles, whereas ZIP transporters increase intracellular zinc availability by promoting extracellular zinc uptake and vesicular zinc release into the cytoplasm. Both ZnT and ZIP transporter families exhibit unique tissue-specific expression, differential responsiveness to dietary zinc deficiency and excess, and differential responsiveness to physiologic stimuli *via* hormones and cytokines (Cousins *et al.*, 2006). In a recent study, low levels of ZnT1 have been observed in mammary gland tumor cells. The zinc concentration in these cells is also higher than that in normal cells, which suggests that zinc transport is misregulated in these proliferating tumor cells, and zinc availability might be essential for tumor cell growth (Lee *et al.*, 2003). In another study, ZIP6 (also known as LIV-1), a breast cancer-associated protein, which belongs to a new subfamily of ZIP transporters, has been found to be associated with estrogen-positive breast cancer and metastasis to lymph nodes (Taylor er la., 2003). Similarly, Kagara *et al.* (Kagara *et al.*, 2007) found that zinc and the transporter ZIP10 were involved in invasive behavior of breast cancer cells. Those studies suggest a positive correlation between zinc or zinc transporters and cancer progression.

[0006] PC still remains a significant health and society problem, and there are no effective treatments. The present invention provides a solution to a longfelt need for PC treatment.

BRIEF SUMMARY OF THE INVENTION

[0007] This invention demonstrates that ZIP4 can serve as a new molecular target for PC. ZIP4 regulates PC growth *via* IL-6/STAT3 pathway, and the genomic profile of ZIP4 facilitates the choice of therapy using an agent that inhibnits ZIP4, such as ZIP4 shRNA and/or ZIP4 shRNA plus gemcitabine, for example.

[0008] One embodiment of the invention is a method of inhibiting proliferation of at least one cancer cell in an individual, comprising delivering to the individual an effective

amount of a composition comprising an agent that inhibits ZIP4. Another embodiment of the invention is a method of treating an individual with cancer, comprising delivering to the individual an effective amount of a composition comprising an agent that inhibits ZIP4.

[0009] In specific embodiments of the invention, the agent that inhibits ZIP4 may comprise nucleic acid. In further embodiment, the nucleic acid comprises RNA. In another embodiment, the RNA is siRNA. In specific instances, the siRNA is shRNA. In further embodiments, the shRNA is SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, or any combination thereof. In another embodiment of the invention, the inhibiting agent specifically targets a specific region of ZIP4. In specific embodiments, this region is the located at 1896 – 1915 bp (SEQ ID NO: 9) in ZIP4 sequence (genbank NM_130849; SEQ ID NO: 1). Although it is targeted to the small region on ZIP4 sequence, the siRNA binding to this region will initiate the whole gene mRNA degradation.

[0010] An embodiment of the invention is the treatment of cancer, or the inhibition of cancer cells. Specific embodiments of the invention include treatment of breast, prostate, lung, bladder, lung, breast, prostate, brain, stomach, colon, spleen, liver, pancreatic, melanoma, head and neck, thyroid, skin, testes, kidney, ovary, cervix, bone, colon cancer, and so forth. or pancreatic cancer. A further embodiment of the invention is the treatment of pancreatic cancer. In some embodiments of the invention, the ZIP4 inhibiting agent only inhibits growth of cancer cells, and not non-cancerous cells.

[0011] The agent that inhibits ZIP4 may be used in combination with other anti-cancer therapies. In one embodiment of the invention, the anti-cancer therapy comprises chemotherapy, radiotherapy, immunotherapy, gene therapy, and/or surgery.

[0012] In embodiments of the invention, the ZIP4 inhibiting agent is delivered to an individual or an individual's cell or cells. In specific embodiments of the invention, the agent is delivered in multiple cycles of treatment. In embodiments of the invention, the delivery is by viral delivery, nanoparticle delivery, virus-like particle deliver, liposomal delivery, or any combination thereof.

[0013] One embodiment of the invention is a kit for cancer treatment, the kit is housed in a suitable container and comprises a first anti-cancer agent that inhibits ZIP4 expression and/or activity. In another embodiment of the invention, the first agent comprises

siRNA. In another embodiment of the invention, the siRNA is shRNA. In a further embodiment of the invention, the siRNA comprises SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, or any combinatino thereof. In a specific embodiment of the invention, the kit also comprises one or more anti cancer agents. In another embodiment of the invention, the anti-cancer agent is a chemotherapeutic agent. In a specific embodiment of the invention, the chemotherapeutic is effective against breast, lung, prostate, and/or pancreatic cancer. In another embodiment, the chemotherapeutic agent is gemcitabine, 5-fluorouracil, cisplatin, irinotecan, paclitaxel, capecitabine, oxaliplatin, streptozocin, or a combination thereof. The kit may additionally comprise one or more radioisotopes. In specific instances, the radioisotope comprises Iodine-131, Iridium-192, Strontium-89, Samarium-153, and/or Boron-10.

[0014] An embodiment of the invention is a method of screening for an agent that inhibits ZIP4, comprising the steps of (a) providing a candidate modulator; (b) admixing the candidate modulator with a respective ZIP4 polynucleotide or polypeptide; and (c) assaying association of the candidate modulator with the respective polynucleotide or polypeptide, wherein when the candidate modulator associates with the respective polynucleotide or polypeptide, said candidate modulator is an agent. Another embodiment of the invention, said association is further defined as binding. In another embodiment, the method further comprises manufacturing the agent. The method may also comprise delivering the agent to an individual that has cancer or that is suspected of having cancer or is at risk for having PC (such as, for exampe, risks due to family history of PC, age, smoking, diet, history of cirrhosis, chronic pancreatitis, and/or diabetes).

[0015] The foregoing has outlined rather broadly the features and technical advantages of the present invention in order that the detailed description of the invention that follows may be better understood. Additional features and advantages of the invention will be described hereinafter which form the subject of the claims of the invention. It should be appreciated by those skilled in the art that the conception and specific embodiment disclosed may be readily utilized as a basis for modifying or designing other structures for carrying out the same purposes of the present invention. It should also be realized by those skilled in the art that such equivalent constructions do not depart from the spirit and scope of the invention as set forth in the appended claims. The novel features which are believed to be characteristic of the invention, both as to its organization and method of operation, together with further objects and

advantages will be better understood from the following description when considered in connection with the accompanying figures. It is to be expressly understood, however, that each of the figures is provided for the purpose of illustration and description only and is not intended as a definition of the limits of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] For a more complete understanding of the present invention, reference is now made to the following descriptions taken in conjunction with the accompanying drawing, in which:

[0017] FIG. 1 shows ZIP4 expression in human pancreatic cancer tissue specimens and cell lines. FIG. 1A is cDNA microarray analysis done by using Affymetrix chips containing 6,800 genes. Microdissected samples from pancreatic adenocarcinoma (Juhasz *et al.*, 2003), chronic pancreatitis (Costello and Franklin, 2006), and normal pancreas (Costello and Franklin, 2006) were analyzed. *, $P < 0.05$. FIG. 1B is the validation of microarray results of ZIP4 mRNA levels in 17 patients. Total RNA was extracted from tissues, and the mRNA levels for ZIP4 were analyzed by real-time PCR and normalized to that of the house keeping gene, β-*actin*. Relative mRNA level is presented as fold increase for both tumor and normal samples. All data shown are the means ± SD of three separate experiments. *, $P < 0.05$. FIG. 1C is immunohistochemical staining of ZIP4 expression in human pancreatic cancer tissue and its normal surrounding tissue. Dark brown color represents positive staining of ZIP4. Arrowheads, normal ductal epithelial cells; arrows, tumor epithelial cells. FIG. 1D shows expression of ZIP4 in human pancreatic cancer cell lines by real-time RT-PCR analysis. The mRNA levels of ZIP4 in eight human pancreatic cancer cell lines were examined by real-time RT-PCR as above. All data shown are the means ± SD of three separate experiments.

[0018] FIG. 2 demonstrates the effect of ZIP4 on MIA CaPa-2 cell proliferation. FIG. 2A is overexpression of ZIP4 mRNA in MIA PaCa-2 cells. mRNA levels in MIA-V and MIA-ZIP4 cells were examined with real-time RT-PCR. Human ZIP4 mRNA levels were normalized to that of human β-*actin*. ZIP4 mRNA levels in MIA-ZIP4 cells were significantly higher than that in MIA-V cells. *, $P < 0.01$. FIG. 2B is overexpression of ZIP4 protein in MIA PaCa-2 cells detected by Western blot. Specific anti-ZIP4 Ab (1:500) was used to probe the protein bands. FIG. 2C shows Zinc concentration in MIA PaCa-2 cells. MIA-V and MIA-ZIP4

cells were treated with 4 μM TPEN for 1 h at 37°C and then incubated with DMEM in the presence of 10 μM $ZnCl_2$ for 5 min before collection. The zinc concentration was examined by ICPMS. *, $P < 0.05$. FIG. 2D is a MTS assay. MIA-V and MIA-ZIP4 cells were seeded in 96-well plates (2×10^3 cells per well) and serum-starved for 24 h before examining the cell proliferation. Absorbance at 490 nm was recorded daily until day 5 after starvation. Data were expressed as the means ± SD of triplicate values. *, $P < 0.05$. FIG. 2E is a zinc-dependent assay. MIA-V and MIA-ZIP4 cells in 96-well plates (2×10^3 cells per well) were treated with 4 μM TPEN for 1 h at 37°C and then incubated with DMEM in the presence of 0, 1, 5, 25, 50, and 125 μM $ZnCl_2$. Absorbance at 490 nm was recorded at day 1 after treatment. Data were expressed as the means ± SD of triplicate values. *, $P < 0.05$.

[0019] FIG. 3 shows the effects of ZIP4 on pancreatic cancer growth in the nude mouse model of s.c. xenograft. FIG. 3A shows MIA-ZIP4 or MIA-V cells (3×10^6) were s.c. inoculated into the right flank of nude mice ($n = 10$ per treatment group). Tumor size was measured weekly for 6 weeks. Tumor volume was calculated by the formula: tumor volume [mm^3] = (length [mm]) × (width [mm])^2 × 0.52. *, $P < 0.01$. A representative s.c. tumor mass from each group was shown in the above the graph. FIG. 3B shows the s.c. tumors that were removed and processed for immunohistochemistry analysis. A monoclonal Ab against Ki67 was used to stain the tissue slides from MIA-V and MIA-ZIP4 groups. s.c. tumors from MIA-ZIP4 group showed much increased cell proliferation by Ki67 staining compared with that of the MIA-V mice. FIG. 3C shows zinc concentration in nude mouse s.c. tumors. The s.c. tumors were removed and homogenized for zinc detection with ICPMS. The average zinc concentration in the MIA-V group and MIA-ZIP4 group was presented. *, $P < 0.05$.

[0020] FIG. 4 demonstrates the effects of ZIP4 on pancreatic tumor progression in the nude mouse model of orthotopic xenograft. FIG. 4A shows the tumor weight. MIA-ZIP4 or MIA-V cells (3×10^6) were orthotopically inoculated into the pancreas of nude mice ($n = 5$ per treatment group). Tumor weight was measured after the mice were euthanized at 7 weeks. *, $P < 0.01$. A representative orthotopic tumor mass from each group is shown in the pictures above the graph. FIG. 4B shows primary tumors. The orthotopic primary tumors were removed, and a representative picture from each group is shown. FIG. 4C are pictures of gross appearance. Pictures of the gross appearance and the primary tumors of each group are shown. FIG. 4D shows zinc concentration in nude mouse primary pancreas tumors. The orthotopic pancreas

tumors were removed and homogenized for zinc detection with ICPMS. The average zinc concentration in the MIA-V group and MIA-ZIP4 group was presented. *, $P < 0.05$.

[0021] FIG. 5 demonstrates ZIP4 expression was silenced by shRNA in ASPC-1 cells. FIG. 5A is real time PCR. Total RNA was extracted from ASPC-shV and ASPC-ZIP4 cells and the mRNA levels for ZIP4 were analyzed by real time PCR, and normalized to that of the house keeping gene, β-actin. Relative mRNA level is presented as fold increase compared with the normal samples. All data shown are the mean±SD of three separate experiments. *$p < 0.05$. FIG. 5B is western blot analysis. Specific anti-ZIP4 Ab (1:500) was used to probe the protein bands.

[0022] FIG. 6 shows the effect of ZIP4 silencing on ASPC-1 cell proliferation, migration, and invasion. FIGS. 6A and 6B show MTS assays. ASPC-shV and ASPC-shZIP4 cells in 96-well plates ($2\times10_3$ cells/well) were treated with 4 μM of TPEN for 1 h at 37°C, and then incubated with DMEM in the presence of (FIG. 6A) 1 and (FIG. 6B) 5 μM $ZnCl_2$. Absorbance at 490 nm was recorded everyday after treatment. Data were expressed as the mean±SD of triplicate values. *$p < 0.05$. FIGS. 6C and D show cell migration and invasion by modified Boyden chamber assay. ASPC-shV and ASPC-shZIP4 cells were trypsinized and resuspended in growth medium (10^6 cells/mL) and added into the upper compartment of a migration chamber (FIG. 6C), or matrigel coated invasion chamber (FIG. 6D), for 24 h. The cells were stained with Calcein-AM. The mean fluorescence reading after scraping of the cells at the top was divided by the reading before removal of the top cells, and the ratio was plotted. Data were expressed as the mean±SD of triplicate values. *$p < 0.05$.

[0023] FIG. 7 shows the effects of ZIP4 silencing on pancreatic cancer growth in the nude mouse model of subcutaneous xenograft. FIG. 7A shows ASPC-shV and ASPC-shZIP4 cells (3×10^6) were subcutaneously inoculated into the right flank of nude mice (n=10 per treatment group). Tumor size was measured weekly for four weeks. Tumor volume was calculated by the formula: tumor volume [mm^3] = (length [mm]) x (width [mm])$^2 \times 0.52$. *$p <$ 0.001. FIG. 7B shows S.C. tumor weight. The above mentioned s.c. tumor was dissected, and the tumor weight was recorded. *$p < 0.05$. A representative s.c. tumor mass from each group was shown in the pictures above the graph.

[0024] FIG. 8 shows the effects of ZIP4 silencing on pancreatic tumor progression and survival in the nude mouse model of orthotopic xenograft. FIG. 8A is tumor weight. MIA-ZIP4 or MIA-V cells (3×10^6) were orthotopically inoculated into the pancreas of nude mice (n=10 per treatment group). Tumor weight was measured after the mice were euthanized at 4 weeks. The orthotopic primary tumors were removed and a representative picture from each group was shown in the picture shown above the graph. FIGS. 8B and C show the orthotopic tumors were removed and processed for H&E staining (FIG. 8B) or Ki67 staining (FIG. 8C). FIG. 8D shows the survival rate. ASPC-shV and ASPC-shZIP4 cells (3×10^6) were orthotopically implanted into the pancreas of nude mice (n=10 per treatment group). Survival rate was examined everyday up to 32 days and was analyzed by the Kaplan-Meier plot and the log-rank test. Survival of mice with ASPC-shZIP4 implantations was substantially longer than that of mice with ASPC-shV implantations ($P = 0.0011$).

[0025] FIG. 9 demonstrates the effects of silencing of ZIP4 in ASPC-1 cells on tumor growth and survival in the xenograft nude mouse model. FIG. 9A is ZIP4 expression in shRNA transfected ASPC-1 cells by Western blot analysis. FIG. 9B shows subcutaneous tumor volume. ASPC-shZIP4 or ASPC-shV cells (3×10^6) were subcutaneously inoculated into nude mice (10 mice per treatment group). Tumor size was measured weekly for 4 weeks. FIG. 9C shows s.c. tumor weight. The above mentioned s.c. tumor were dissected, and the tumor weight was recorded. FIG. 9D demonstrates orthotopic tumor weight. ASPC-shV and ASPC-shZIP4 cells (3×10^6) were orthotopically inoculated into the pancreas of nude mice (n=5 per treatment group). Tumor weight was measured after the mice were euthanized at 4 weeks. FIG. 9E shows survival rate. ASPC-shV and ASPC-shZIP4 cells (3×10^6) were orthotopically inoculated into the pancreas of nude mice (n=10 per treatment group). Survival rate was examined everyday for 32 days.

[0026] FIG. 10 shows ZIP4 is over-expressed in human PC tissue specimens. Two representative tumor samples are shown. Low power field (100x) shows carcinomatous ducts and cells along with adjacent benign (#1) and PanIN ducts (#2). Black arrows, positive staining of ZIP4 on the membrane of cancer cells in the pancreatic adenocarcinoma (1-1, 2-1). Open arrows, negative staining of ZIP4 in the benign (1-2) and PanIN lesions (2-2).

[0027] FIG. 11 shows phosphorylation of STAT3 in MIA-ZIP4 cells. Stable MIA-V and MIA-ZIP4 cells were examined for the expression and phosphorylation of STAT3 protein.

Briefly, cells lysates were extracted and probed with anti-p(tyr)STAT3 (1:5000), and anti-STAT3 (1:2500) Abs as described previously (Bharadwaj *et al.*, 2007).

[0028] FIG. 12 demonstrates ZIP4 upregulates the expression of cyclin D1 in PC xenografts. FIG. 12A is a microarray analysis. Cyclin D1 mRNA was upregulated in MIA-ZIP4 group compared with that in the MIA-V group. FIG. 12B shows Cyclin D1 mRNA levels in MIA-V and MIA-ZIP4 groups in orthotopic xenografts. The microarray data was confirmed by real time RT PCR.

[0029] FIG. 13 shows that ZIP4 upregulates the expression of IL-6 in PC xenografts. FIG. 12A is an orthotopic model. FIG. 12B is a subcutaneous model. IL-6 mRNA was upregulated in MIA-ZIP4 group compared with that in the MIA-V group by real time PCR.

[0030] FIG. 14 shows zinc-dependent PC cell proliferation. MIA-V and MIA-ZIP4 cells in 96-well plates (2×10^3 cells/well) were treated with 4 μM of TPEN for 1 h at 37°C to chelate the existing zinc ions, and then incubated with DMEM in the presence of 0, 1, 5, 25, 50, and 125 μM $ZnCl_2$. MTS assay was used to determine the cell proliferation. Absorbance at 490 nm was recorded at day 1 after treatment. Data were expressed as the mean±SD of triplicate values. *$p < 0.05$.

[0031] FIG. 15 demonstrates silencing of PDX-1 by shRNA inhibits tumor growth in xenografts of PC. Panc-1 tumor-bearing SCID mice were treated with 35ug liposome/ huPDX-1 shRNA intravenously once every two weeks, for a total of three cycles. pSUPER vector control treatments served as control. On day 30 following the last gene delivery, mice were sacrificed and necropsy was performed for assessment of tumor volume. Tumor size was measured and compared using Student's *t* test.

[0032] FIG. 16 shows high levels of K-Ras activity causes acinar cells to undergo metaplasia to ductal cells and to form mPanINs, invasive and metastatic PC. FIG. 16A shows mPanIN lesions developed and progressed from mPanIN-1 to mPanIN-3 with age. FIG. 16B shows ductal adenocarcinoma developed in mouse expressing active K-Ras in pancreatic acinar cells after 4 months. FIG 16C shows metastasis in the liver.

[0033] FIG. 17 demonstrates mZIP4 was highly expressed in mouse PC, but not normal pancreas. The expression of mZIP4 was examined in pancreas tissues from Kras

transgenic mouse using immunohistochemical staining with mouse ZIP4 antibody. Two representative mouse tissues were shown in this figure. Arrow indicates positive staining of mZIP4 in tumor epithelial cells. N, normal ductal epithelial cells; and T, tumor epithelial cells.

[0034] FIG. 18 is an embodiment of a working model for ZIP4-mediated signal transduction in PC.

DETAILED DESCRIPTION OF THE INVENTION

I. DEFINITIONS

[0035] In keeping with long-standing patent law convention, the words "a" and "an" when used in the present specification in concert with the word comprising, including the claims, denote "one or more." Some embodiments of the invention may consist of or consist essentially of one or more elements, method steps, and/or methods of the invention. It is contemplated that any method or composition described herein can be implemented with respect to any other method or composition described herein.

[0036] The term "synergistic" or "synergistically" as used herein refers to the addition of two reactants that may or may not react in the same pathway with each other, from which the resulting product of the reaction proceeds to a further extent than one of skill in the art would predict. In a specific embodiment, two compounds act synergistically when the result achieved upon using them in combination is greater than the sum of the results of the compounds when used separately.

[0037] The phrase " effective amount" as used herein means that amount of a compound, material, or composition comprising a compound of the present invention that is effective for producing some desired effect, *e.g.*, halting the growth of, reducing the size of, and/or causing apoptosis in a cancer cell. In one embodiment, the effective amount is enough to reduce or eliminate at least one cancer cell. One of skill in the art recognizes that an amount may be considered effective even if the cancer cell is not totally eradicated but decreased partially. For example, the spread of the cancer may be halted or reduced, a side effect from the cancer may be partially reduced or completed eliminated, and so forth. The effective amount may also be a therapeutically effective amount.

[0038] The terms "inhibit," "inhibitory," or "inhibitor" as used herein refers to one or more molecules that interfere at least in part with the growth or activity of the molecule or cell it inhibits. The inhibition of a cancer cell may be the inhibition of growth of at least one cancer cell.

[0039] As used herein, "treat" and all its forms and tenses (including, for example, treat, treating, treated, and treatment) refer to both therapeutic treatment and prophylactic or preventative treatment. Those in need thereof of treatment include those already with a pathological condition of the invention (including, for example, a cancer) as well as those in which a pathological condition of the invention is to be prevented. In certain embodiments, the terms "treating" and "treatment" as used herein refer to administering to a subject a therapeutically effective amount of a composition so that the subject has an improvement in the disease or condition. The improvement is any observable or measurable improvement. Thus, one of skill in the art realizes that a treatment may improve the individual's condition, but may not be a complete cure of the disease. Treating may also comprise treating subjects at risk of developing a disease and/or condition of the invention.

[0040] As used herein the term "metastatic" (and all other forms and tenses, including, for example, metastasis, metastasize, etc.) when used alone or in conjunction with cancer refers to the spread of a cancer from one part of the body to another, unless otherwise indicated by the use or context. Typically, a tumor formed by cells that have spread is called a "metastatic tumor" or a "metastasis." The metastatic tumor contains cells that are like those in the original (primary) tumor.

[0041] As used herein, an "individual" is an appropriate individual for the method of the present invention. A subject may be a mammal and in specific embodiments is any member of the higher vertebrate class Mammalia, including humans; characterized by live birth, body hair, and mammary glands in the female that secrete milk for feeding the young. Additionally, mammals are characterized by their ability to maintain a constant body temperature despite changing climatic conditions. Examples of mammals are humans, cats, dogs, cows, mice, rats, horses, sheep, pigs and chimpanzees. Subjects may also be referred to as "patients" or "subjects".

1. Zinc and Zinc Transport

[0042] Zinc is an essential trace element and catalytic/structural component utilized by many enzymes and transcription factors that contain zinc finger motifs (Mao *et al.*, 2007; Wang *et al.*, 2002). Zinc is essential for growth, and zinc deficiency in animals leads to growth retardation, impaired DNA synthesis, and severe dermatitis (King and Cousins, 2005). Recent studies have shown that zinc availability is also important for tumor growth and progression because zinc is a critical component for many enzymes involved in hypoxia, angiogenesis, cell proliferation, and metastasis of cancers (Juhasz *et al.*, 2003). On the other hand, high concentrations of zinc are toxic so cells have evolved a complex system to maintain the balance of zinc uptake, intracellular storage, and efflux (Kim *et al.*, 2004; Liuzzi and Cousins, 2004). Two solute-linked carrier (SLC) gene families were identified as zinc transporters, SLC30 which encodes for ZnT proteins, and SLC39 which encodes for ZIP proteins (Liuzzi and Cousins, 2004 Guerinot, 2000; Eide, 2004). They appear to have opposite roles in cellular zinc homeostasis. ZnT transporters reduce intracellular zinc availability by promoting zinc efflux from cells or into intracellular vesicles, while ZIP transporters increase intracellular zinc availability by promoting extracellular zinc uptake and vesicular zinc release into the cytoplasm. Both ZnT and ZIP transporter families exhibit unique tissue-specific expression, differential responsiveness to dietary zinc deficiency and excess, and differential responsiveness to physiologic stimuli via hormones and cytokines (Cousins *et al.*, 2006). Altered expression of zinc transporters is associated with many diseases, including cancers. Recently, low levels of ZnT1 have been observed in mammary gland tumor cells. The zinc concentration in these cells are also higher than that in normal cells, which suggests that zinc transport is misregulated in these proliferating tumor cells, and zinc availability might be essential for tumor cell growth (Lee *et al.*, 2003). In another study, ZIP6 (also known as LIV- 1), a breast cancer-associated protein, which belongs to a new subfamily (LZT subfamily) of ZIP transporters, has been associated with estrogen-positive breast cancer and metastasis to lymph nodes (Taylor *et al.*, 2003). Similarly, Kagara *et al.* found that zinc and the transporter ZIP10 were involved in invasive behavior of breast cancer cells (Kagara *et al.*, 2007). Those reports thus suggest a positive correlation between zinc, zinc transporters and cancer progression. ZIP4, encoded by the SLC39A4 gene, plays an important role in maintaining the cellular zinc level by facilitating uptake of dietary zinc into intestinal epithelial cells, and releasing zinc from vesicular compartments (Mao *et al.*, 2007; Kim *et al.*, 2004; Liuzzi *et al.*, 2004). Mutations in SLC39A4 gene are thought to be the cause of a genetic

disorder of zinc deficiency, acrodermatitis enteropathica (AE) (Wang and Cousins, 2005; Kury *et al.*, 2002).

[0043] Previous studies have shown that zinc regulates the activity of many zinc finger transcription factors such as metal-responsive transcription factor-1 (MTF-1), cAMP response element-binding protein (CREB), and CREB binding protein (CBP). Zinc also controls the expression and secretion of many cytokines such as IL-6 (Mariani *et al.*, 2007; Liuzzi *et al.*, 2005; Liuzzi *et al.*, 2006; Rink and Kirchner, 2000; Driessen *et al.*, 1987; Salas and Kirchner, 1987; Scuderi, 1990). On the other hand, IL-6 may also exert a positive feedback to regulate the expression of zinc transporters (Liuzzi *et al.*, 2005). High zinc concentration is toxic to the cells, therefore, cells have evolved a complex system to maintain the balance of zinc uptake, intracellular storage, and efflux (Dufner-Beattie *et al.*, 2003). These cells also have a higher than normal concentration of zinc, which suggests a misregulation of zinc transport in these proliferating tumor cells, and zinc availability might be essential for tumor cell growth (Liuzzi and Cousins, 2004; Lee *et al.*, 2003). In another study, LIV-1 (also called ZIP6), the breast cancer-associated protein, which belongs to a new subfamily of ZIP transporters, has been found to be associated with estrogen-positive breast cancer and its metastatic spread to the regional lymph nodes, suggesting a positive correlation between the zinc importer and cancer progression (Taylor *et al.*, 2003; Taylor *et al.*, 2004).

2. Pancreatic Cancer

[0044] As the fourth leading cause of cancer-related deaths in North America, pancreatic cancer has the highest fatality rate of all cancers. Survival statistics are poor, because there are no reliable tests for early diagnosis and no effective therapies for the metastatic form of pancreatic cancer (Landis *et al.*, 1998; Torrisani and Buscail, 2002; Warshaw and Fernandez-del Castillo, 1992). By the time diagnosis is made, the disease has usually spread to distant sites of the body.

[0045] Representative symptoms of pancreatic cancer include pain in the abdomen and back, loss of appetite, bloating, diarrhea or fatty bowel movements, and jaundice, for example. Diagnosis may be made on physical exam, abdominal ultrasound, and/or abdominal computed tomography, for example. A biopsy may be performed either percutaneously or endoscopically. Treatment is usually through chemotherapy, radiation therapy, and surgery. The

most commonly used chemotherapies are gemcitabine, fluorouracil, and capecitabine. The present invention may be employed with any comventional treatment of PC, for example.

3. Pancreatic Cancer and Diet

[0046] Recently, the diet and nutrition prevention have brought more attention in cancer therapy including pancreatic cancer. Epidemiology studies on patient diet and nutrition uptake have indicated that pancreatic cancer patients do better on a largely plant-based diet that contains low zinc. Tested subjects who ate the most processed meat or most red meat (high zinc diet) had a 68% or 50% greater risk of having pancreatic cancer, respectively, compared with those who ate the least processed meat and red meat. These results provide a diet recommendation for pancreatic cancer patients with low zinc diet emphasizing more vegetables and less meat (Nothlings *et al.*, 2005).

[0047] The diet and nutrition prevention have brought more attention lately in cancer therapy including PC. Epidemiology studies on patient diet and nutrition uptake have indicated that PC patients do best on a largely plant-based diet. Those patients tend to metabolize most efficiently with specific nutrients that are found in fruits, vegetables, nuts, and with minimal to no animal protein. A meat diet is loaded with minerals such as phosphorous and zinc, which have the opposite effect. In one embodiment of the invention, an individual with a diet high in zinc has an increased risk of pancreatic cancer. In another embodiment of the invention, the invention is used in combination with a low zinc diet.

4. ZIP4

[0048] ZIP4, encoded by the SLC39A4 gene, plays an important role in maintaining the cellular zinc level by uptaking dietary zinc into intestinal epithelial cells and releasing zinc from vesicular compartments (Mao *et al.*, 2007; Kim *et al.*, 2004; Liuzzi *et al.*, 2004). Mutations in the SLC39A4 gene are thought to be the reason for a genetic disorder of zinc-deficiency acrodermatitis enteropathica (AE) (Wang *et al.*, 2002; Kury *et al.*, 2002). Recently, molecular profiling studies found that ZIP4 mRNA levels were markedly higher in human pancreatic cancer (Logsdon *et al.*, 2003).

[0049] Solute carrier family 39 (zinc transporter), member 4 [Homo sapiens] (SLC39A4), is also called AEZ, ZIP4, FLJ20327, MGC74741. It is located on chromosome 8, specifically 8q24.3. This gene encodes a member of the zinc/iron-regulated transporter-like protein (ZIP) family. The transmembrane protein is required for zinc uptake in the intestine. Mutations in this gene result in acrodermatitis enteropathica, a rare inherited defect in the absorption of dietary zinc. Multiple transcript variants encoding different isoforms have been found for this gene. Genbank accession NM_130849.2 (SEQ ID NO: 1)is encode for isoform 2, which has 12 exons. Genbank accession NM_017767.2 (SEQ ID NO:4) is encode for isoform 1, which contains 11 exons, a shorter version of the isoform 2 is SEQ ID NO: 3.

[0050] ZIP4 maintains the cellular zinc level. The importance of zinc is also demonstrated by a genetic disorder of zinc deficiency, acrodermatitis enteropathica (AE), which is caused by mutations in ZIP4 (encoded by *SLC39A4* gene), a specific zinc importer for dietary zinc absorption, which is mapped on the same chromosome (8q24.3) with AE (Wang *et al.*, 2002; Kury *et al.*, 2002). ZIP4 belongs to a broader ZIP family, which stands for Zrt-, Irt-like protein, representing the first identified members of the transporter family (Guerinot, 2000; Eide, 2004). ZIP4 plays an important role in maintaining the cellular zinc level by uptaking dietary zinc into the cells and is regulated at multiple levels in response to changes in zinc availability, and zinc-regulated trafficking and endocytosis of ZIP4 is a key mechanism controlling dietary zinc absorption and cellular zinc homeostasis (Kim *et al.*, 2004). The examples indicate that ZIP4 was over-expressed in human PC, and aberrant expression of ZIP4 contributed to PC growth (Li *et al.*, 2007). Considering the importance of zinc in many enzymes for tumor growth and metastasis, it is of great interest to study the role of ZIP4 in PC pathogenesis.

[0051] Methods of the present invention particularly concern inhibiting proliferation of deleterious cells, such as cancer cells, although in alternative cases the proliferation of other types of cells is inhibited, such as cells involved in restenosis, for example. The proliferation of the cell is inhibited at least in part by the inhibition of the expression and/or activity and/or by increasing the degradation or rate of degradation of ZIP4. The inhibition of these targets may be by any means, including by targeting polynucleotides that encode ZIP4, for example, by targeting polypeptides encoded thereby, or by targeting both.

[0052] In particular cases, one or more specific agents are employed to inhibit ZIP4. The agent may be of any suitable kind so long as it is effective in inhibiting ZIP4 at least

partially. The agent may include a polynucleotide, which may be referred to as nucleic acid; a polypeptide, which may be a protein, in some cases; a peptide; a small molecule; or a mixture thereof. The agent may be a natural agent, a synthetic agent, or a mixture thereof. The present invention encompasses methods of identifying such an agent, including by assaying libraries of small molecules, by assaying inhibitory nucleic acids, or both, for example. Any assay to identify one or more agents is suitable for the invention so long as a ZIP4 inhibitory agent is identifiable.

[0053] In specific embodiments, the agent that inhibits ZIP4 includes nucleic acid, such as DNA, RNA, or a mixture thereof. The RNA may be of any kind, such as RNA suitable for RNA interference. In particular cases, siRNA is employed as the inhibitory agent, and the skilled artisan may employ the exemplary siRNA molecules provided herein or may similarly generate other suitable siRNAs to inhibited ZIP4. shRNA may also be used to inhibit ZIP4.

II. RNAi AND siRNA EMBODIMENTS

[0054] RNA interference (also referred to as "RNA-mediated interference"; RNAi) is a mechanism by which gene expression can be reduced or eliminated. Double stranded RNA (dsRNA) or single stranded RNA has been observed to mediate the reduction, which is a multi-step process (for details of single stranded RNA methods and compositions see Martinez *et al.*, 2002, for example). dsRNA activates post-transcriptional gene expression surveillance mechanisms that appear to function to defend cells from virus infection and transposon activity (Fire *et al.*, 1998; Grishok *et al.*, 2000; Ketting *et al.*, 1999; Lin *et al.*, 1999; Montgomery *et al.*, 1998; Sharp *et al.*, 2000; Tabara *et al.*, 1999). Activation of these mechanisms targets mature, dsRNA-complementary mRNA for destruction. RNAi offers major experimental advantages for study of gene function. These advantages include a very high specificity, ease of movement across cell membranes, and prolonged down-regulation of the targeted gene. (Fire *et al.*, 1998; Grishok *et al.*, 2000; Ketting *et al.*, 1999; Lin *et al.*, 1999; Montgomery *et al.*, 1998; Sharp, 1999; Sharp *et al.*, 2000; Tabara *et al.*, 1999). Moreover, dsRNA has been shown to silence genes in a wide range of systems, including plants, protozoans, fungi, *C. elegans*, *Trypanasoma, Drosophila*, and mammals (Grishok *et al.*, 2000; Sharp, 1999; Sharp *et al.*, 2000; Elbashir *et al.*, 2001).

[0055] RNA interference (RNAi) is a naturally occurring intracellular pathway in which small RNA molecules bind to mRNA to trigger its degradation. Small interfering RNA

(siRNA) is highly effective and specific in knocking down the target genes, and has significant advantages over traditional methods such as chemical inhibitors and dominant negatives. Therefore, siRNA therapy represents a novel and promising treatment for PC. The examples indicate a strong inhibitory effect of ZIP4 siRNA in PC growth. siRNA can be introduced into the cells by using either chemically synthesized siRNA oligonucleotides (oligos), or vector based siRNA (short hairpin RNA, shRNA), which allows long lasting and more stable gene silencing. Liposomes are commonly used carriers delivering the siRNA with better transfection efficiency and protecting it from degradation. In combination with standard chemotherapy, siRNA therapy can also reduce the chemoresistance of certain cancers, demonstrating the potential of siRNA therapy in treating many malignant diseases (Huang et la., 2008).

[0056] The term "small hairpin siRNA," "short hairpin siRNA," or "shRNAs," as used herein, refers to siRNAs composed of a single strand of RNA that possesses regions of self-complementarity that cause the single strand to fold back upon itself and form a hairpin-like structure with an intramolecular duplexed region containing at least 19 basepairs. Importantly, because they are single-stranded, shRNAs can be readily expressed from single expression cassettes. All siRNA embodiments of the invention also include shRNAs.

[0057] SiRNAs are small RNAs that do not significantly induce the antiviral response common among vertebrate cells but that do induce target mRNA degradation *via* the RNAi pathway. The term siRNA refers to RNA molecules that have either at least one double stranded region or at least one single stranded region and possess the ability to effect RNAi. It is specifically contemplated that siRNA may refer to RNA molecules that have at least one double stranded region and possess the ability to effect RNAi. Mixtures or pools of dsRNAs (siRNAs) may be generated by various methods including chemical synthesis, enzymatic synthesis of multiple templates, digestion of long dsRNAs by a nuclease with RNAse III domains, and the like. A “pool” or “cocktail” refers to a composition that contains at least two siRNA molecules that have different selectivity with respect to each other, but are directed to the same target gene. Two or more siRNA molecules that have different selectivity with respect to each other, but are directed to the same or different target gene(s) are defined as different siRNAs. Different siRNAs may overlap in sequence, contain two sequences that are contiguous or non-contiguous in the target gene. In some embodiments, a pool contains at least or at most 3, 4, 5, 6, 7, 8, 9, 10 or more siRNA molecules. An “siRNA directed to” a particular region or target gene means that

a particular siRNA includes sequences that results in the reduction or elimination of expression of the target gene, *i.e.*, the siRNA is targeted to the region or gene. The pool in some embodiments includes one or more control siRNA molecules. In other embodiments a control siRNA molecule is not included in the pool. A pool of siRNA molecules may also contain various candidate siRNA molecules that do not reduce or eliminate expression of a target gene.

[0058] Some of the uses for RNAi include implementing therapeutics and diagnostics, identifying genes that are essential for a particular biological pathway, identifying disease-causing genes, and studying structure function relationships. As with other types of gene inhibitory compounds, such as antisense and triplex forming oligonucleotides, tracking these potential drugs *in vivo* and *in vitro* is important for drug development, pharmacokinetics, biodistribution, macro and microimaging metabolism and for gaining a basic understanding of how these compounds behave and function. siRNAs have high specificity and may be used to knock out the expression of a single allele of a dominantly mutated diseased gene.

[0059] In certain embodiments of the invention, ZIP4 is targeted with one or more siRNA molecules. Thus, in specific embodiments a cancer cell or a cell suspected of being or becoming cancerous is provided with one or more siRNAs directed against ZIP4. In particular embodiments, an individual with cancer, suspected of having cancer, at a high risk for cancer, or susceptible to cancer is administered one or more siRNA molecules directed against ZIP4.

[0060] The present invention includes methods and compositions for introducing multiple siRNAs targeting different regions of a gene that typically can greatly improve the likelihood that the expression of the target gene will be reduced. The inventors have found that the different candidate siRNAs or siRNAs do not interfere with the activities of others in the mixture and that in fact, there appears to be some synergy between the siRNAs. This is applicable not only to siRNAs but to DNA constructs designed to express siRNAs (Brummelkamp, 2002). Certain embodiments of the invention alleviate the need to screen or optimize candidate siRNAs. To determine the functionality of a candidate siRNA, some siRNA must be screened, verified, and/or optimized. As used herein, a "candidate siRNA" is an siRNA that has not been tested for its functionality as an siRNA. It is also contemplated that siRNAs may be single or double stranded RNA molecules.

[0061] In some embodiments of the invention, methods are employed wherein multiple therapeutic RNAs are employed, each of which reduce the expression of a target gene to some degree, as well as the presence of some dsRNAs, which do not effect target gene expression, may be administered as a pool without interference between members of the pool and may result in an additive or synergistic reduction in target gene expression. Thus, the present invention is directed to compositions and methods involving generation and utilization of pools or mixtures of small, double-stranded RNA molecules that effect, trigger, or induce RNAi more effectively. RNAi is mediated by an RNA-induced silencing complex (RISC), which associates (specifically binds one or more RISC components) with dsRNA pools of the invention and guides the dsRNA to its target mRNA through base-pairing interactions. Once the dsRNA is base-paired with its mRNA target, nucleases cleave the mRNA.

[0062] In certain embodiments of the invention, one or more siRNAs or dsRNAs can be introduced into a cell to activate the RNAi pathway. In other embodiments, various individual siRNAs or dsRNAs with different sequences may be co-transfected simultaneously to effectively produce a pool or mixture of dsRNAs within a transfected cell(s). The effects of multiple siRNAs are typically additive and may be synergistic in some cases. siRNA may also be used in combination with an additional cancer therapy.

[0063] In some embodiments, the invention concerns a siRNA or dsRNA that is capable of triggering RNA interference, a process by which a particular RNA sequence is destroyed (also referred to as gene silencing). siRNA are dsRNA molecules that are 100 bases or fewer in length (or have 100 basepairs or fewer in its complementarity region). A dsRNA may be 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 225, 250, 275, 300, 325, 350. 375, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, or 1000 nucleotides or more in length. In certain embodiments, siRNA may be approximately 21 to 25 nucleotides in length. In some cases, it has about a two nucleotide 3' overhang and a 5' phosphate. The particular RNA sequence is targeted as a result of the complementarity between the dsRNA and the particular RNA sequence. It will be understood that siRNA or dsRNA of the invention can effect at least about a 20, 30, 40, 50, 60, 70, 80, 90 percent or more reduction of expression of a targeted RNA in a cell. dsRNA of the invention (the term "dsRNA" will be understood to include "siRNA" and/or "candidate siRNA") is distinct and distinguishable from antisense and ribozyme molecules by virtue of the

ability to trigger RNAi. Structurally, dsRNA molecules for RNAi comprise at least one region of complementarity within the RNA molecule. The complementary (also referred to as "complementarity") region comprises at least or at most about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 441, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690, 700, 710, 720, 730, 740, 750, 760, 770, 780, 790, 800, 810, 820, 830, 840, 850, 860, 870, 880, 890, 900, 910, 920, 930, 940, 950, 960, 970, 980, 990, or 1000 contiguous bases. In some embodiments, long dsRNA are employed in which "long" refers to dsRNA that are 1000 bases or longer (or 1000 basepairs or longer in complementarity region). The term "dsRNA" includes "long dsRNA", "intermediate dsRNA" or "small dsRNA" (lengths of 2 to 100 bases or basepairs in complementarity region) unless otherwise indicated.

[0064] It is specifically contemplated that a dsRNA may be a molecule comprising two separate RNA strands in which one strand has at least one region complementary to a region on the other strand. Alternatively, a dsRNA includes a molecule that is single stranded yet has at least one complementarity region as described above (see Sui *et al.*, 2002 and Brummelkamp *et al.*, 2002 in which a single strand with a hairpin loop is used as a dsRNA for RNAi). For convenience, lengths of dsRNA may be referred to in terms of bases, which simply refers to the length of a single strand or in terms of basepairs, which refers to the length of the complementarity region. It is specifically contemplated that embodiments discussed herein with respect to a dsRNA comprised of two strands are contemplated for use with respect to a dsRNA comprising a single strand, and vice versa. In a two-stranded dsRNA molecule, the strand that has a sequence that is complementary to the targeted mRNA is referred to as the "antisense strand" and the strand with a sequence identical to the targeted mRNA is referred to as the "sense strand." Similarly, with a dsRNA comprising only a single strand, it is contemplated that the "antisense region" has the sequence complementary to the targeted mRNA, while the "sense region" has the sequence identical to the targeted mRNA. Furthermore, it will be understood that sense and antisense region, like sense and antisense strands, are complementary (*i.e.*, can specifically hybridize) to each other.

[0065] Strands or regions that are complementary may or may not be 100% complementary ("completely or fully complementary"). It is contemplated that sequences that are "complementary" include sequences that are at least about 50% complementary, and may be at least about 50%, 60%, 70%, 80%, or 90% complementary. In the range of about 50% to 70% complementarity, such sequences may be referred to as "very complementary," while the range of greater than about 70% to less than complete complementarity can be referred to as "highly complementary." Unless otherwise specified, sequences that are "complementary" include sequences that are "very complementary," "highly complementary," and "fully complementary." It is also contemplated that any embodiment discussed herein with respect to "complementary" strands or region can be employed with specifically "fully complementary," "highly complementary," and/or "very complementary" strands or regions, and vice versa. Thus, it is contemplated that in some instances, as demonstrated in the Examples, that siRNA generated from sequence based on one organism may be used in a different organism to achieve RNAi of the cognate target gene. In other words, siRNA generated from a dsRNA that corresponds to a human gene may be used in a mouse cell if there is the requisite complementarity, as described above. Ultimately, the requisite threshold level of complementarity to achieve RNAi is dictated by functional capability.

[0066] It is specifically contemplated that there may be mismatches in the complementary strands or regions. Mismatches may number at most or at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 residues or more, depending on the length of the complementarity region.

[0067] The single RNA strand or each of two complementary double strands of a dsRNA molecule may be of at least or at most the following lengths: 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 441, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690, 700, 710, 720, 730, 740, 750, 760, 770, 780, 790, 800, 810, 820, 830, 840, 850, 860, 870, 880, 890, 900, 910, 920, 930, 940, 950, 960, 970,

980, 990, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, 2100, 2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900, 3000, 31, 3200, 3300, 3400, 3500, 3600, 3700, 3800, 3900, 4000, 4100, 4200, 4300, 4400, 4500, 4600, 4700, 4800, 4900, 5000, 6000, 7000, 8000, 9000, 10000 or more (including the full-length of a particular's gene's mRNA without the poly-A tail) bases or basepairs. If the dsRNA is comprised of two separate strands, the two strands may be the same length or different lengths. If the dsRNA is a single strand, in addition to the complementarity region, the strand may have 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 or more bases on either or both ends (5' and/or 3') or as forming a hairpin loop between the complementarity regions.

[0068] Furthermore, it is contemplated that siRNA or the longer dsRNA template may be labeled. The label may be fluorescent, radioactive, enzymatic, or colorimetric, for example. It is contemplated that a dsRNA may have one label attached to it or it may have more than one label attached to it. When more than one label is attached to a dsRNA, the labels may be the same or be different. If the labels are different, they may appear as different colors when visualized. The label may be on at least one end and/or it may be internal. Furthermore, there may be a label on each end of a single stranded molecule or on each end of a dsRNA made of two separate strands. The end may be the 3' and/or the 5' end of the nucleic acid. A label may be on the sense strand or the sense end of a single strand (end that is closer to sense region as opposed to antisense region), or it may be on the antisense strand or antisense end of a single strand (end that is closer to antisense region as opposed to sense region). In some cases, a strand is labeled on a particular nucleotide (G, A, U, or C). When two or more differentially colored labels are employed, fluorescent resonance energy transfer (FRET) techniques may be employed to characterize the dsRNA.

[0069] Labels contemplated for use in several embodiments may be non-radioactive. In many embodiments of the invention, the labels are fluorescent, though they may be enzymatic, radioactive, or positron emitters. Fluorescent labels that may be used include, but are not limited to, BODIPY, Alexa Fluor, fluorescein, Oregon Green, tetramethylrhodamine, Texas Red, rhodamine, cyanine dye, or derivatives thereof, for example. The labels may also

more specifically be Alexa 350, Alexa 430, AMCA, BODIPY 630/650, BODIPY 650/665, BODIPY-FL, BODIPY-R6G, BODIPY-TMR, BODIPY-TRX, Cascade Blue, Cy3, Cy5, DAPI, 6-FAM, Fluorescein Isothiocyanate, HEX, 6-JOE, Oregon Green 488, Oregon Green 500, Oregon Green 514, Pacific Blue, REG, Rhodamine Green, Rhodamine Red, Renographin, ROX, SYPRO, TAMRA, TET, Tetramethylrhodamine, and/or Texas Red, for example. A labeling reagent is a composition that comprises a label and that can be incubated with the nucleic acid to effect labeling of the nucleic acid under appropriate conditions. In some embodiments, the labeling reagent comprises an alkylating agent and a dye, such as a fluorescent dye. In some embodiments, a labeling reagent comprises an alkylating agent and a fluorescent dye such as Cy3, Cy5, or fluorescein (FAM). In still further embodiments, the labeling reagent is also incubated with a labeling buffer, which may be any buffer compatible with physiological function (*i.e.*, buffers that is not toxic or harmful to a cell or cell component) (termed "physiological buffer").

[0070] In some embodiments of the invention, a dsRNA has one or more non-natural nucleotides, such as a modified residue or a derivative or analog of a natural nucleotide. Any modified residue, derivative or analog may be used to the extent that it does not eliminate or substantially reduce (by at least 50%) RNAi activity of the dsRNA.

[0071] A person of ordinary skill in the art is well aware of achieving hybridization of complementary regions or molecules. Such methods typically involve heat and slow cooling of temperature during incubation.

[0072] Any cell that undergoes RNAi can be employed in methods of the invention. The cell may be a eukaryotic cell, mammalian cell such as a primate, rodent, rabbit, canine, feline, equine, or human cell, for example.

[0073] In some embodiments of the invention, there are methods of reducing the expression of a target gene in a cell. Such methods involve the compositions described above, including the embodiments described for RNase III, dsRNA, and siRNA. In various embodiments of the invention, reduction or elimination of expression of one or more target genes may be accomplished by the a) obtaining one or more siRNA or dsRNA molecules corresponding one or more target genes and b) transfecting the respective siRNA or dsRNA molecules corresponding to the one or more target genes into a cell.

[0074] In some methods of the invention, siRNA and/or candidate siRNA molecules or template nucleic acids may be isolated or purified prior to their being used in a subsequent step. siRNA and/or candidate siRNA molecules may be isolated or purified prior to introduction into a cell. "Introduction" into a cell includes known methods of transfection, transduction, infection and other methods for introducing an expression vector or a heterologous nucleic acid into a cell. A template nucleic acid or amplification primer may be isolated or purified prior to it being transcribed or amplified. Isolation or purification can be performed by a number of methods known to those of skill in the art with respect to nucleic acids. In some embodiments, a gel, such as an agarose or acrylamide gel, is employed to isolate the siRNA and/or candidate siRNA.

[0075] In some methods of the invention dsRNA is obtained by transcribing each strand of the dsRNA from one or more cDNA (or DNA or RNA) encoding the strands *in vitro*. It is contemplated that a single template nucleic acid molecule may be used to transcribe a single RNA strand that has at least one region of complementarity (and is thus double-stranded under conditions of hybridization) or it may be used to transcribe two separate complementary RNA molecules. Alternatively, more than one template nucleic acid molecule may be transcribed to generate two separate RNA strands that are complementary to one another and capable of forming a dsRNA.

[0076] Additional methods involve isolating the transcribed strand(s) and/or incubating the strand(s) under conditions that allow the strand(s) to hybridize to their complementary strands (or regions if a single strand is employed).

[0077] Nucleic acid templates may be generated by a number of methods well known to those of skill in the art. In some embodiments the template, such as a cDNA, is synthesized through amplification or it may be a nucleic acid segment in or from a plasmid that harbors the template.

[0078] In various embodiments, siRNAs are encoded by expression constructs. The expression constructs may be obtained and introduced into a cell. Once introduced into the cell the expression construct is transcribed to produce various siRNAs. Expression constructs include nucleic acids that provide for the transcription of a particular nucleic acid. Expression constructs include plasmid DNA, linear expression elements, circular expression elements, viral

expression constructs, and the like, all of which are contemplated as being used in the compositions and methods of the present invention. In certain embodiments at least about 2, 3, 4, 5, 6, 7, 8, 9, 10 or more siRNA molecules are encoded by a single expression construct. Expression of the siRNA molecules may be independently controlled by at least about 2, 3, 4, 5, 6, 7, 8, 9, 10 or more promoter elements. In certain embodiments, at least about 2, 3, 4, 5, 6, 7, 8, 9, 10 or more expression constructs may introduced into the cell. Each expression construct may encode 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more siRNA molecules. In certain embodiments siRNA molecules may be encoded as expression domains. Expression domains include a transcription control element, which may or may not be independent of other control or promoter elements; a nucleic acid encoding an siRNA; and optionally a transcriptional termination element. In other words, an siRNA cocktail or pool may be encoded by a single or multiple expression constructs. In particular embodiments the expression construct is a plasmid expression construct.

[0079] Other methods of the invention also concern transcribing a strand or strands of a dsRNA using a promoter that can be employed *in vitro* or outside a cell, such as a prokaryotic promoter. In some embodiments, the prokaryotic promoter is a bacterial promoter or a bacteriophage promoter. It is specifically contemplated that dsRNA strands are transcribed with SP6, T3, or T7 polymerase.

III. NUCLEIC ACIDS OF THE INVENTION

[0080] Certain embodiments of the present invention concern nucleic acids, including ZIP4 nucleic acids and siRNAs that inhibit ZIP4 nucleic acids. The following description will employ ZIP4 as an exemplary embodiment. In certain aspects, a ZIP4 nucleic acid comprises a wild-type or a mutant ZIP4 nucleic acid. An exemplary ZIP4 nucleic acid sequence is comprised in SEQ ID NO:1 (GenBank® Accession Number NM_130849) and is provided for any application, including to facilitate identifying a suitable siRNA sequence for the invention, for example. In particular aspects, a ZIP4 nucleic acid encodes for or comprises a transcribed nucleic acid, although in other cases the nucleic acid comprises a fragment of a ZIP4 nucleic acid that encodes a polypeptide or comprises a siRNA sequence. In other aspects, a ZIP4 nucleic acid comprises a nucleic acid segment of SEQ ID NO:1, or a biologically functional equivalent thereof. In particular aspects, a ZIP4 nucleic acid encodes a protein, polypeptide, peptide, although in embodiments such as with siRNA the molecule may be too short to effectively encode a gene product.

[0081] The term "nucleic acid" is well known in the art. A "nucleic acid" as used herein will generally refer to a molecule (*i.e.*, a strand) of DNA, RNA or a derivative or analog thereof, comprising a nucleobase. A nucleobase includes, for example, a naturally occurring purine or pyrimidine base found in DNA (*e.g.*, an adenine "A," a guanine "G," a thymine "T" or a cytosine "C") or RNA (*e.g.*, an A, a G, an uracil "U" or a C). The term "nucleic acid" encompass the terms "oligonucleotide" and "polynucleotide," each as a subgenus of the term "nucleic acid." In some embodiments, the term "oligonucleotide" refers to a molecule of between about 3 and about 100 nucleobases in length. In some embodiments, the term "polynucleotide" refers to at least one molecule of greater than about 100 nucleobases in length.

[0082] These definitions generally refer to a single-stranded molecule, but in specific embodiments will also encompass an additional strand that is partially, substantially or fully complementary to the single-stranded molecule. Thus, a nucleic acid may encompass a double-stranded molecule or a triple-stranded molecule that comprises one or more complementary strand(s) or "complement(s)" of a particular sequence comprising a molecule. As used herein, a single stranded nucleic acid may be denoted by the prefix "ss," a double stranded nucleic acid by the prefix "ds," and a triple stranded nucleic acid by the prefix "ts."

1. Nucleobases

[0083] As used herein a "nucleobase" refers to a heterocyclic base, such as for example a naturally occurring nucleobase (*i.e.*, an A, T, G, C or U) found in at least one naturally occurring nucleic acid (*i.e.*, DNA and RNA), and naturally or non-naturally occurring derivative(s) and analogs of such a nucleobase. A nucleobase generally can form one or more hydrogen bonds ("anneal" or "hybridize") with at least one naturally occurring nucleobase in manner that may substitute for naturally occurring nucleobase pairing (*e.g.*, the hydrogen bonding between A and T, G and C, and A and U).

[0084] "Purine" and/or "pyrimidine" nucleobase(s) encompass naturally occurring purine and/or pyrimidine nucleobases and also derivative(s) and analog(s) thereof, including but not limited to, those a purine or pyrimidine substituted by one or more of an alkyl, caboxyalkyl, amino, hydroxyl, halogen (*i.e.*, fluoro, chloro, bromo, or iodo), thiol or alkylthiol moeity. Preferred alkyl (*e.g.*, alkyl, caboxyalkyl, etc.) moeities comprise of from about 1, about 2, about 3, about 4, about 5, to about 6 carbon atoms. Other non-limiting examples of a purine or pyrimidine include a deazapurine, a 2,6-diaminopurine, a 5-fluorouracil, a xanthine, a

hypoxanthine, a 8-bromoguanine, a 8-chloroguanine, a bromothymine, a 8-aminoguanine, a 8-hydroxyguanine, a 8-methylguanine, a 8-thioguanine, an azaguanine, a 2-aminopurine, a 5-ethylcytosine, a 5-methylcyosine, a 5-bromouracil, a 5-ethyluracil, a 5-iodouracil, a 5-chlorouracil, a 5-propyluracil, a thiouracil, a 2-methyladenine, a methylthioadenine, a N,N-diemethyladenine, an azaadenines, a 8-bromoadenine, a 8-hydroxyadenine, a 6-hydroxyaminopurine, a 6-thiopurine, a 4-(6-aminohexyl/cytosine), and the like. A nucleobase may be comprised in a nucleoside or nucleotide, using any chemical or natural synthesis method described herein or known to one of ordinary skill in the art.

2. Nucleosides

[0085] As used herein, a "nucleoside" refers to an individual chemical unit comprising a nucleobase covalently attached to a nucleobase linker moiety. A non-limiting example of a "nucleobase linker moiety" is a sugar comprising 5-carbon atoms (*i.e.*, a "5-carbon sugar"), including but not limited to a deoxyribose, a ribose, an arabinose, or a derivative or an analog of a 5-carbon sugar. Non-limiting examples of a derivative or an analog of a 5-carbon sugar include a 2'-fluoro-2'-deoxyribose or a carbocyclic sugar where a carbon is substituted for an oxygen atom in the sugar ring.

[0086] Different types of covalent attachment(s) of a nucleobase to a nucleobase linker moiety are known in the art. By way of non-limiting example, a nucleoside comprising a purine (*i.e.*, A or G) or a 7-deazapurine nucleobase typically covalently attaches the 9 position of a purine or a 7-deazapurine to the 1'-position of a 5-carbon sugar. In another non-limiting example, a nucleoside comprising a pyrimidine nucleobase (*i.e.*, C, T or U) typically covalently attaches a 1 position of a pyrimidine to a 1'-position of a 5-carbon sugar (Kornberg and Baker, 1992).

3. Nucleotides

[0087] As used herein, a "nucleotide" refers to a nucleoside further comprising a "backbone moiety". A backbone moiety generally covalently attaches a nucleotide to another molecule comprising a nucleotide, or to another nucleotide to form a nucleic acid. The "backbone moiety" in naturally occurring nucleotides typically comprises a phosphorus moiety, which is covalently attached to a 5-carbon sugar. The attachment of the backbone moiety

typically occurs at either the 3'- or 5'-position of the 5-carbon sugar. However, other types of attachments are known in the art, particularly when a nucleotide comprises derivatives or analogs of a naturally occurring 5-carbon sugar or phosphorus moiety.

4. Nucleic Acid Analogs

[0088] A nucleic acid may comprise, or be composed entirely of, a derivative or analog of a nucleobase, a nucleobase linker moiety and/or backbone moiety that may be present in a naturally occurring nucleic acid. As used herein a "derivative" refers to a chemically modified or altered form of a naturally occurring molecule, while the terms "mimic" or "analog" refer to a molecule that may or may not structurally resemble a naturally occurring molecule or moiety, but possesses similar functions. As used herein, a "moiety" generally refers to a smaller chemical or molecular component of a larger chemical or molecular structure. Nucleobase, nucleoside and nucleotide analogs or derivatives are well known in the art, and have been described (see for example, Scheit, 1980, incorporated herein by reference).

[0089] Additional non-limiting examples of nucleosides, nucleotides or nucleic acids comprising 5-carbon sugar and/or backbone moiety derivatives or analogs, include those in U.S. Patent No. 5,681,947 which describes oligonucleotides comprising purine derivatives that form triple helixes with and/or prevent expression of dsDNA; U.S. Patents 5,652,099 and 5,763,167 which describe nucleic acids incorporating fluorescent analogs of nucleosides found in DNA or RNA, particularly for use as flourescent nucleic acids probes; U.S. Patent 5,614,617 which describes oligonucleotide analogs with substitutions on pyrimidine rings that possess enhanced nuclease stability; U.S. Patents 5,670,663, 5,872,232 and 5,859,221 which describe oligonucleotide analogs with modified 5-carbon sugars (*i.e.*, modified 2'-deoxyfuranosyl moieties) used in nucleic acid detection; U.S. Patent 5,446,137 which describes oligonucleotides comprising at least one 5-carbon sugar moiety substituted at the 4' position with a substituent other than hydrogen that can be used in hybridization assays; U.S. Patent 5,886,165 which describes oligonucleotides with both deoxyribonucleotides with 3'-5' internucleotide linkages and ribonucleotides with 2'-5' internucleotide linkages; U.S. Patent 5,714,606 which describes a modified internucleotide linkage wherein a 3'-position oxygen of the internucleotide linkage is replaced by a carbon to enhance the nuclease resistance of nucleic acids; U.S. Patent 5,672,697 which describes oligonucleotides containing one or more 5' methylene phosphonate internucleotide linkages that enhance nuclease resistance; U.S. Patents 5,466,786 and 5,792,847

which describe the linkage of a substituent moeity which may comprise a drug or label to the 2' carbon of an oligonucleotide to provide enhanced nuclease stability and ability to deliver drugs or detection moieties; U.S. Patent 5,223,618 which describes oligonucleotide analogs with a 2 or 3 carbon backbone linkage attaching the 4' position and 3' position of adjacent 5-carbon sugar moiety to enhanced cellular uptake, resistance to nucleases and hybridization to target RNA; U.S. Patent 5,470,967 which describes oligonucleotides comprising at least one sulfamate or sulfamide internucleotide linkage that are useful as nucleic acid hybridization probe; U.S. Patents 5,378,825, 5,777,092, 5,623,070, 5,610,289 and 5,602,240 which describe oligonucleotides with three or four atom linker moeity replacing phosphodiester backbone moeity used for improved nuclease resistance, cellular uptake and regulating RNA expression; U.S. Patent 5,858,988 which describes hydrophobic carrier agent attached to the 2'-O position of oligonuceotides to enhanced their membrane permeability and stability; U.S. Patent 5,214,136 which describes oligonucleotides conjugaged to anthraquinone at the 5' terminus that possess enhanced hybridization to DNA or RNA; enhanced stability to nucleases; U.S. Patent 5,700,922 which describes PNA-DNA-PNA chimeras wherein the DNA comprises 2'-deoxy-erythro-pentofuranosyl nucleotides for enhanced nuclease resistance, binding affinity, and ability to activate RNase H; and U.S. Patent 5,708,154 which describes RNA linked to a DNA to form a DNA-RNA hybrid.

5. Polyether and Peptide Nucleic Acids

[0090] In certain embodiments, it is contemplated that a nucleic acid comprising a derivative or analog of a nucleoside or nucleotide may be used in the methods and compositions of the invention. A non-limiting example is a "polyether nucleic acid", described in U.S. Patent Serial No. 5,908,845, incorporated herein by reference. In a polyether nucleic acid, one or more nucleobases are linked to chiral carbon atoms in a polyether backbone.

[0091] Another non-limiting example is a "peptide nucleic acid", also known as a "PNA", "peptide-based nucleic acid analog" or "PENAM", described in U.S. Patent Serial Nos. 5,786,461, 5891,625, 5,773,571, 5,766,855, 5,736,336, 5,719,262, 5,714,331, 5,539,082, and WO 92/20702, each of which is incorporated herein by reference. Peptide nucleic acids generally have enhanced sequence specificity, binding properties, and resistance to enzymatic degradation in comparison to molecules such as DNA and RNA (Egholm *et al.*, 1993; PCT/EP/01219). A peptide nucleic acid generally comprises one or more nucleotides or

nucleosides that comprise a nucleobase moiety, a nucleobase linker moeity that is not a 5-carbon sugar, and/or a backbone moiety that is not a phosphate backbone moiety. Examples of nucleobase linker moieties described for PNAs include aza nitrogen atoms, amido and/or ureido tethers (see for example, U.S. Patent No. 5,539,082). Examples of backbone moieties described for PNAs include an aminoethylglycine, polyamide, polyethyl, polythioamide, polysulfinamide or polysulfonamide backbone moiety.

[0092] In certain embodiments, a nucleic acid analogue such as a peptide nucleic acid may be used to inhibit nucleic acid amplification, such as in PCR, to reduce false positives and discriminate between single base mutants, as described in U.S. Patent Serial No. 5891,625. Other modifications and uses of nucleic acid analogs are known in the art, and are encompassed by the invention. In a non-limiting example, U.S. Patent 5,786,461 describes PNAs with amino acid side chains attached to the PNA backbone to enhance solubility of the molecule. In another example, the cellular uptake property of PNAs is increased by attachment of a lipophilic group. U.S. application Ser. No. 117,363 describes several alkylamino moeities used to enhance cellular uptake of a PNA. Another example is described in U.S. Patent Nos. 5,766,855, 5,719,262, 5,714,331 and 5,736,336, which describe PNAs comprising naturally and non-naturally occurring nucleobases and alkylamine side chains that provide improvements in sequence specificity, solubility and/or binding affinity relative to a naturally occurring nucleic acid.

6. Preparation of Nucleic Acids

[0093] The present invention concerns various nucleic acids in different embodiments of the invention. For example, there are a variety of ways to generate a dsRNA that can function as an siRNA or can be used as a substrate for a polypeptide with RNase III activity to generate siRNAs. In some embodiments, dsRNA is created by transcribing a DNA template. The DNA template may be comprised in a vector or it may be a non-vector template. Alternatively, a dsRNA may be created by hybridizing two synthetic, complementary RNA molecules or hybridizing a single synthetic RNA molecule with at least one intramolecular complementarity region. Such nucleic acids may be made by any technique known to one of ordinary skill in the art, such as for example, chemical synthesis, enzymatic production or biological production.

7. Purification of Nucleic Acids

[0094] A nucleic acid may be purified on polyacrylamide gels, cesium chloride centrifugation gradients, or by any other means known to one of ordinary skill in the art (see for example, Sambrook *et al.*, 1989, incorporated herein by reference).

[0095] In certain aspect, the present invention concerns a nucleic acid that is an isolated nucleic acid. As used herein, the term "isolated nucleic acid" refers to a nucleic acid molecule (*e.g.*, an RNA or DNA molecule) that has been isolated free of, or is otherwise free of, the bulk of the total genomic and transcribed nucleic acids of one or more cells. In certain embodiments, "isolated nucleic acid" refers to a nucleic acid that has been isolated free of, or is otherwise free of, bulk of cellular components or *in vitro* reaction components such as for example, macromolecules such as lipids or proteins, small biological molecules, and the like.

8. Nucleic Acid Segments

[0096] In certain embodiments, the nucleic acid is a nucleic acid segment. As used herein, the term "nucleic acid segment," are smaller fragments of a nucleic acid, such as for non-limiting example, those that encode only part of the ZIP4 peptide or polypeptide sequence. Thus, a "nucleic acid segment" may comprise any part of a gene sequence, of from about 2 nucleotides to the full length of the ZIP4 peptide or polypeptide encoding region.

[0097] Various nucleic acid segments may be designed based on a particular nucleic acid sequence, and may be of any length. By assigning numeric values to a sequence, for example, the first residue is 1, the second residue is 2, *etc.*, an algorithm defining all nucleic acid segments can be created:

[0098] n to n + y

[0099] where n is an integer from 1 to the last number of the sequence and y is the length of the nucleic acid segment minus one, where n + y does not exceed the last number of the sequence. Thus, for a 10 mer, the nucleic acid segments correspond to bases 1 to 10, 2 to 11, 3 to 12 ... and so on. For a 15-mer, the nucleic acid segments correspond to bases 1 to 15, 2 to 16, 3 to 17 ... and so on. For a 20-mer, the nucleic segments correspond to bases 1 to 20, 2 to 21, 3 to 22 ... and so on. In certain embodiments, the nucleic acid segment may be a probe or primer.

As used herein, a "probe" generally refers to a nucleic acid used in a detection method or composition. As used herein, a "primer" generally refers to a nucleic acid used in an extension or amplification method or composition. Exemplary ZIP4 primers for real time PCR are sense 5'-ATGTCAGGAGCGGGTCTTGC-3' (SEQ ID NO: 5); and anti-sense 5'-GCTGCTGTGCTGCTGGAAC-3'(SEQ ID NO: 6).

9. Nucleic Acid Complements

[0100] The present invention also encompasses a nucleic acid that is complementary at least in part to a ZIP4 nucleic acid. In particular embodiments the invention encompasses a nucleic acid or a nucleic acid segment complementary to part or all of the sequence set forth in SEQ ID NO:1. A nucleic acid is "complement(s)" or is "complementary" to another nucleic acid when it is capable of base-pairing with another nucleic acid according to the standard Watson-Crick, Hoogsteen or reverse Hoogsteen binding complementarity rules. As used herein "another nucleic acid" may refer to a separate molecule or a spatial separated sequence of the same molecule.

[0101] As used herein, the term "complementary" or "complement(s)" also refers to a nucleic acid comprising a sequence of consecutive nucleobases or semiconsecutive nucleobases (*e.g.*, one or more nucleobase moieties are not present in the molecule) capable of hybridizing to another nucleic acid strand or duplex even if less than all the nucleobases do not base pair with a counterpart nucleobase. In certain embodiments, a "complementary" nucleic acid comprises a sequence in which about 70%, about 71%, about 72%, about 73%, about 74%, about 75%, about 76%, about 77%, about 77%, about 78%, about 79%, about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, to about 100%, and any range derivable therein, of the nucleobase sequence is capable of base-pairing with a single or double stranded nucleic acid molecule during hybridization. In certain embodiments, the term "complementary" refers to a nucleic acid that may hybridize to another nucleic acid strand or duplex in stringent conditions, as would be understood by one of ordinary skill in the art.

[0102] In certain embodiments, a "partly complementary" nucleic acid comprises a sequence that may hybridize in low stringency conditions to a single or double stranded nucleic

acid, or contains a sequence in which less than about 70% of the nucleobase sequence is capable of base-pairing with a single or double stranded nucleic acid molecule during hybridization.

10. Hybridization

[0103] As used herein, "hybridization", "hybridizes" or "capable of hybridizing" is understood to mean the forming of a double or triple stranded molecule or a molecule with partial double or triple stranded nature. The term “anneal” as used herein is synonymous with “hybridize.” The term "hybridization", "hybridize(s)" or "capable of hybridizing" encompasses the terms "stringent condition(s)" or "high stringency" and the terms "low stringency" or "low stringency condition(s)."

[0104] As used herein "stringent condition(s)" or "high stringency" are those conditions that allow hybridization between or within one or more nucleic acid strand(s) containing complementary sequence(s), but precludes hybridization of random sequences. Stringent conditions tolerate little, if any, mismatch between a nucleic acid and a target strand. Such conditions are well known to those of ordinary skill in the art, and are preferred for applications requiring high selectivity. Non-limiting applications include isolating a nucleic acid, such as a gene or a nucleic acid segment thereof, or detecting at least one specific mRNA transcript or a nucleic acid segment thereof, and the like.

[0105] Stringent conditions may comprise low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.15 M NaCl at temperatures of about 50°C to about 70°C. It is understood that the temperature and ionic strength of a desired stringency are determined in part by the length of the particular nucleic acid(s), the length and nucleobase content of the target sequence(s), the charge composition of the nucleic acid(s), and to the presence or concentration of formamide, tetramethylammonium chloride or other solvent(s) in a hybridization mixture.

[0106] It is also understood that these ranges, compositions and conditions for hybridization are mentioned by way of non-limiting examples only, and that the desired stringency for a particular hybridization reaction is often determined empirically by comparison to one or more positive or negative controls. Depending on the application envisioned it is preferred to employ varying conditions of hybridization to achieve varying degrees of selectivity

of a nucleic acid towards a target sequence. In a non-limiting example, identification or isolation of a related target nucleic acid that does not hybridize to a nucleic acid under stringent conditions may be achieved by hybridization at low temperature and/or high ionic strength. Such conditions are termed "low stringency" or "low stringency conditions", and non-limiting examples of low stringency include hybridization performed at about 0.15 M to about 0.9 M NaCl at a temperature range of about 20°C to about 50°C. Of course, it is within the skill of one in the art to further modify the low or high stringency conditions to suite a particular application.

[0107] In certain embodiments, the wild-type sequence of ZIP4 may be employed for identification of inhibitors of ZIP4, such as siRNAs. As used herein "wild-type" refers to the naturally occurring sequence of a nucleic acid at a genetic locus in the genome of an organism, or a sequence transcribed or translated from such a nucleic acid. Thus, the term "wild-type" also may refer to an amino acid sequence encoded by a nucleic acid. As a genetic locus may have more than one sequence or alleles in a population of individuals, the term "wild-type" encompasses all such naturally occurring allele(s). As used herein the term "polymorphic" means that variation exists (*i.e.*, two or more alleles exist) at a genetic locus in the individuals of a population. As used herein "mutant" refers to a change in the sequence of a nucleic acid or its encoded protein, polypeptide or peptide that is the result of the hand of man.

[0108] The present invention also concerns the isolation or creation of a recombinant construct or a recombinant host cell through the application of recombinant nucleic acid technology known to those of skill in the art or as described herein. A recombinant construct or host cell may comprise a ZIP4 nucleic acid or inhibitor thereof, and may express a ZIP4 protein, polypeptide or peptide, or at least one biologically functional equivalent thereof, although in alternative embodiments the recombinant construct or host cells comprises a nucleic acid that is too short to encode a protein, polypeptide, or peptide.

[0109] Herein certain embodiments, a "gene" refers to a nucleic acid that is transcribed. In certain aspects, the gene includes regulatory sequences involved in transcription, or message production or composition. In particular embodiments, the gene comprises transcribed sequences that encode for a protein, polypeptide or peptide. As will be understood by those in the art, this function term "gene" includes both genomic sequences, RNA or cDNA sequences or smaller engineered nucleic acid segments, including nucleic acid segments of a non-transcribed part of a gene, including but not limited to the non-transcribed promoter or

enhancer regions of a gene. Smaller engineered gene nucleic acid segments may express, or may be adapted to express using nucleic acid manipulation technology, proteins, polypeptides, domains, peptides, fusion proteins, mutants and/or such like.

[0110] "Isolated substantially away from other coding sequences" means that the gene of interest forms the significant part of the coding region of the nucleic acid, or that the nucleic acid does not contain large portions of naturally-occurring coding nucleic acids, such as large chromosomal fragments, other functional genes, RNA or cDNA coding regions. Of course, this refers to the nucleic acid as originally isolated, and does not exclude genes or coding regions later added to the nucleic acid by the hand of man.

[0111] The nucleic acid(s) of the present invention, regardless of the length of the sequence itself, may be combined with other nucleic acid sequences, including but not limited to, promoters, enhancers, polyadenylation signals, restriction enzyme sites, multiple cloning sites, coding segments, and the like, to create one or more nucleic acid construct(s). As used herein, a "nucleic acid construct" is a nucleic acid engeneered or altered by the hand of man, and generally comprises one or more nucleic acid sequences organized by the hand of man.

[0112] In a non-limiting example, one or more nucleic acid constructs may be prepared that include a contiguous stretch of nucleotides identical to or complementary to part or all of SEQ ID NO:1. A nucleic acid construct may be about 3, about 5, about 8, about 10 to about 14, or about 15, about 20, about 30, about 40, about 50, about 100, about 200, about 500, about 1,000, about 2,000, about 3,000, about 5,000, about 10,000, about 15,000, about 20,000, about 30,000, about 50,000, about 100,000, about 250,000, about 500,000, about 750,000, to about 1,000,000 nucleotides in length, as well as constructs of greater size, up to and including chromosomal sizes (including all intermediate lengths and intermediate ranges), given the advent of nucleic acids constructs such as a yeast artificial chromosome are known to those of ordinary skill in the art. It will be readily understood that "intermediate lengths" and "intermediate ranges", as used herein, means any length or range including or between the quoted values (*i.e.*, all integers including and between such values). Non-limiting examples of intermediate lengths include about 11, about 12, about 13, about 16, about 17, about 18, about 19, etc.; about 21, about 22, about 23, etc.; about 31, about 32, etc.; about 51, about 52, about 53, etc.; about 101, about 102, about 103, etc.; about 151, about 152, about 153, etc.; about 1,001, about 1002, etc,; about 50,001, about 50,002, etc; about 750,001, about 750,002, etc.; about 1,000,001, about

1,000,002, etc. Non-limiting examples of intermediate ranges include about 3 to about 32, about 150 to about 500,001, about 3,032 to about 7,145, about 5,000 to about 15,000, about 20,007 to about 1,000,003, etc.

[0113] In certain embodiments, the nucleic acid construct is a recombinant vector. In particular embodiments, the invention concerns one or more recombinant vector(s) comprising nucleic acid sequences that is capable of encoding an ZIP4 protein, polypeptide or peptide that includes within its amino acid sequence a contiguous amino acid sequence in accordance with, or essentially as set forth in, SEQ ID NO:2. In particular aspects, the recombinant vectors are DNA vectors, although in alternative embodiments the vectors comprise RNA vectors.

[0114] The term "a sequence essentially as set forth in SEQ ID NO:1" encompasses nucleic acids, nucleic acid segments, and genes that comprise part or all of the nucleic acid sequences as set forth in SEQ ID NO:1.

[0115] In certain other embodiments, the invention concerns at least one recombinant vector that includes within its sequence a nucleic acid sequence essentially as set forth in SEQ ID NO:1, or a fragment thereof.

[0116] It will also be understood that amino acid sequences or nucleic acid sequences may include additional residues, such as additional N or C terminal amino acids or 5' or 3' sequences, or various combinations thereof, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the maintenance of biological protein, polypeptide or peptide activity where expression of a proteinaceous composition is concerned. The addition of terminal sequences particularly applies to nucleic acid sequences that may, for example, include various non-coding sequences flanking either of the 5' and/or 3' portions of the coding region or may include various internal sequences, *i.e.*, introns, which are known to occur within genes.

[0117] Excepting intronic and flanking regions, and allowing for the degeneracy of the genetic code, nucleic acid sequences that have between about 70% and about 79%; or more preferably, between about 80% and about 89%; or even more particularly, between about 90% and about 99%; of nucleotides that are identical to the nucleotides of SEQ ID NO:1 will be nucleic acid sequences that are "essentially as set forth in SEQ ID NO:1".

[0118] It will also be understood that this invention is not limited to the particular nucleic acid or amino acid sequences of SEQ ID NO:1 or SEQ ID NO:2, respectively. Recombinant vectors and isolated nucleic acid segments may therefore variously include these coding regions themselves, coding regions bearing selected alterations or modifications in the basic coding region, and they may encode larger polypeptides or peptides that nevertheless include such coding regions or may encode biologically functional equivalent proteins, polypeptide or peptides that have variant amino acids sequences.

[0119] Changes designed by man may be introduced to the nucleic acids of the invention through the application of site-directed mutagenesis techniques, for example.

IV. NUCLEIC ACID-BASED EXPRESSION SYSTEMS

[0120] In certain aspects of the invention, an agent comprising a nucleic acid that inhibits ZIP4 is employed. Such an agent may be comprised within an expression system, such as on a vector, although alternatively the agent is not comprised within an expression system. In other aspects of the invention, the ZIP4 target sequence to which the agent is directed is utilized within an expression system, such as for agent-identifying or agent-generating purposes, for example.

1. Vectors

[0121] Nucleic acids of the invention, particularly DNA templates or DNA constructs for siRNA expression, may be produced recombinantly. Protein and polypeptides may be encoded by a nucleic acid molecule comprised in a vector. The term "vector" is used to refer to a carrier nucleic acid molecule into which a nucleic acid sequence can be inserted for introduction into a cell where it can be replicated. A nucleic acid sequence can be "exogenous," which means that it is foreign to the cell into which the vector is being introduced or that the sequence is homologous to a sequence in the cell but in a position within the host cell nucleic acid in which the sequence is ordinarily not found. Vectors include plasmids, cosmids, viruses (bacteriophage, animal viruses, and plant viruses), and artificial chromosomes (*e.g.*, YACs). One of skill in the art would be well equipped to construct a vector through standard recombinant techniques (see, for example, Maniatis *et al.*, 1988 and Ausubel *et al.*, 1994, both incorporated herein by reference).

[0122] The term "expression vector" refers to any type of genetic construct comprising a nucleic acid coding for a RNA capable of being transcribed. In some cases, RNA

molecules are then translated into a protein, polypeptide, or peptide. In other cases, these sequences are not translated, for example, in the production of antisense molecules or ribozymes. Expression vectors can contain a variety of "control sequences," which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operably linked coding sequence in a particular host cell. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well and are described infra.

2. Promoters and Enhancers

[0123] A "promoter" is a control sequence that is a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind, such as RNA polymerase and other transcription factors, to initiate the specific transcription a nucleic acid sequence. The phrases "operatively positioned," "operatively linked," "under control," and "under transcriptional control" mean that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and/or expression of that sequence.

[0124] A promoter generally comprises a sequence that functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as, for example, the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation. Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30 110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. To bring a coding sequence "under the control of" a promoter, one positions the 5′ end of the transcription initiation site of the transcriptional reading frame "downstream" of (i.e., 3′ of) the chosen promoter. The "upstream" promoter stimulates transcription of the DNA and promotes expression of the encoded RNA.

[0125] The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before

activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription. A promoter may or may not be used in conjunction with an "enhancer," which refers to a cis-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

[0126] A promoter may be one naturally associated with a nucleic acid sequence, as may be obtained by isolating the 5′ non-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as "endogenous." Similarly, an enhancer may be one naturally associated with a nucleic acid sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding nucleic acid segment under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with a nucleic acid sequence in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a nucleic acid sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other virus, or prokaryotic or eukaryotic cell, and promoters or enhancers not "naturally occurring," *i.e.*, containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. For example, promoters that are most commonly used in recombinant DNA construction include the β lactamase (penicillinase), lactose and tryptophan (trp) promoter systems. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR™, in connection with the compositions disclosed herein (see U.S. Patent Nos. 4,683,202 and 5,928,906, each incorporated herein by reference). Furthermore, it is contemplated the control sequences that direct transcription and/or expression of sequences within non-nuclear organelles such as mitochondria, chloroplasts, and the like, can be employed as well.

[0127] Naturally, it will be important to employ a promoter and/or enhancer that effectively directs the expression of the DNA segment in the organelle, cell type, tissue, organ, or organism chosen for expression including for PC. Those of skill in the art of molecular biology generally know the use of promoters, enhancers, and cell type combinations for protein expression, (see, for example Sambrook *et al.* 1989, incorporated herein by reference). The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the

appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins and/or peptides. The promoter may be heterologous or endogenous.

[0128] Additionally any promoter/enhancer combination (as per, for example, the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

[0129] The identity of tissue-specific promoters or elements, as well as assays to characterize their activity, is well known to those of skill in the art. Nonlimiting examples of such regions include the human LIMK2 gene (Nomoto *et al.* 1999), the somatostatin receptor 2 gene (Kraus *et al.*, 1998), murine epididymal retinoic acid-binding gene (Lareyre *et al.*, 1999), human CD4 (Zhao-Emonet *et al.*, 1998), mouse alpha2 (XI) collagen (Tsumaki, *et al.*, 1998), D1A dopamine receptor gene (Lee, *et al.*, 1997), insulin-like growth factor II (Wu *et al.*, 1997), and human platelet endothelial cell adhesion molecule-1 (Almendro *et al.*, 1996).

3. Initiation Signals and Internal Ribosome Binding Sites

[0130] A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon or adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be "in-frame" with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements.

[0131] In certain embodiments of the invention, the use of internal ribosome entry sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5´ methylated Cap dependent translation and

begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message (see U.S. Patent Nos. 5,925,565 and 5,935,819, each herein incorporated by reference).

4. Multiple Cloning Sites

[0132] Vectors can include a multiple cloning site (MCS), which is a nucleic acid region that contains multiple restriction enzyme sites, any of which can be used in conjunction with standard recombinant technology to digest the vector (see, for example, Carbonelli *et al.*, 1999, Levenson *et al.*, 1998, and Cocea, 1997, incorporated herein by reference.) "Restriction enzyme digestion" refers to catalytic cleavage of a nucleic acid molecule with an enzyme that functions only at specific locations in a nucleic acid molecule. Many of these restriction enzymes are commercially available. Use of such enzymes is widely understood by those of skill in the art. Frequently, a vector is linearized or fragmented using a restriction enzyme that cuts within the MCS to enable exogenous sequences to be ligated to the vector. "Ligation" refers to the process of forming phosphodiester bonds between two nucleic acid fragments, which may or may not be contiguous with each other. Techniques involving restriction enzymes and ligation reactions are well known to those of skill in the art of recombinant technology.

5. Splicing Sites

[0133] Most transcribed eukaryotic RNA molecules will undergo RNA splicing to remove introns from the primary transcripts. Vectors containing genomic eukaryotic sequences may require donor and/or acceptor splicing sites to ensure proper processing of the transcript for protein expression (see, for example, Chandler *et al.*, 1997, herein incorporated by reference.)

6. Termination Signals

[0134] The vectors or constructs of the present invention will generally comprise at least one termination signal. A "termination signal" or "terminator" is comprised of the DNA sequences involved in specific termination of an RNA transcript by an RNA polymerase. Thus, in certain embodiments a termination signal that ends the production of an RNA transcript is contemplated. A terminator may be necessary *in vivo* to achieve desirable message levels.

[0135] In eukaryotic systems, the terminator region may also comprise specific DNA sequences that permit site-specific cleavage of the new transcript so as to expose a polyadenylation site. This signals a specialized endogenous polymerase to add a stretch of about 200 A residues (polyA) to the 3' end of the transcript. RNA molecules modified with this polyA tail appear to more stable and are translated more efficiently. Thus, in other embodiments involving eukaryotes, it is preferred that that terminator comprises a signal for the cleavage of the RNA, and it is more preferred that the terminator signal promotes polyadenylation of the message. The terminator and/or polyadenylation site elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

[0136] Terminators contemplated for use in the invention include any known terminator of transcription described herein or known to one of ordinary skill in the art, including but not limited to, for example, the termination sequences of genes, such as for example the bovine growth hormone terminator or viral termination sequences, such as for example the SV40 terminator. In certain embodiments, the termination signal may be a lack of transcribable or translatable sequence, such as due to a sequence truncation.

7. Polyadenylation Signals

[0137] In expression, particularly eukaryotic expression, one will typically include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. Preferred embodiments include the SV40 polyadenylation signal or the bovine growth hormone polyadenylation signal, convenient and known to function well in various target cells. Polyadenylation may increase the stability of the transcript or may facilitate cytoplasmic transport.

8. Origins of Replication

[0138] In order to propagate a vector in a host cell, it may contain one or more origins of replication sites (often termed "ori"), which is a specific nucleic acid sequence at which replication is initiated. Alternatively an autonomously replicating sequence (ARS) can be employed if the host cell is yeast.

9. Selectable and Screenable Markers

[0139] In certain embodiments of the invention, cells containing a nucleic acid construct of the present invention may be identified *in vitro* or *in vivo* by including a marker in the expression vector. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression vector. Generally, a selectable marker is one that confers a property that allows for selection. A positive selectable marker is one in which the presence of the marker allows for its selection, while a negative selectable marker is one in which its presence prevents its selection. An example of a positive selectable marker is a drug resistance marker.

[0140] Usually the inclusion of a drug selection marker aids in the cloning and identification of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. In addition to markers conferring a phenotype that allows for the discrimination of transformants based on the implementation of conditions, other types of markers including screenable markers such as GFP, whose basis is colorimetric analysis, are also contemplated. Alternatively, screenable enzymes such as herpes simplex virus thymidine kinase (tk) or chloramphenicol acetyltransferase (CAT) may be utilized. One of skill in the art would also know how to employ immunologic markers, possibly in conjunction with FACS analysis. The marker used is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable and screenable markers are well known to one of skill in the art.

10. Plasmid Vectors

[0141] In certain embodiments, a plasmid vector is contemplated for use to transform a host cell. In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. In a non-limiting example, *E. coli* is often transformed using derivatives of pBR322, a plasmid derived from an *E. coli* species. pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, for example, promoters which can be used by the microbial organism for expression of its own proteins.

[0142] In addition, phage vectors containing replicon and control sequences that are compatible with the host microorganism can be used as transforming vectors in connection with these hosts. For example, the phage lambda GEMTM 11 may be utilized in making a recombinant phage vector which can be used to transform host cells, such as, for example, *E. coli* LE392.

[0143] Further useful plasmid vectors include pIN vectors (Inouye *et al.*, 1985); and pGEX vectors, for use in generating glutathione S transferase (GST) soluble fusion proteins for later purification and separation or cleavage. Other suitable fusion proteins are those with β galactosidase, ubiquitin, and the like.

[0144] Bacterial host cells, for example, *E. coli*, comprising the expression vector, are grown in any of a number of suitable media, for example, LB. The expression of the recombinant protein in certain vectors may be induced, as would be understood by those of skill in the art, by contacting a host cell with an agent specific for certain promoters, *e.g.*, by adding IPTG to the media or by switching incubation to a higher temperature. After culturing the bacteria for a further period, generally of between 2 and 24 h, the cells are collected by centrifugation and washed to remove residual media.

11. Viral Vectors

[0145] The ability of certain viruses to infect cells or enter cells *via* receptor mediated endocytosis, and to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign nucleic acids into cells (*e.g.*, mammalian cells). Non-limiting examples of virus vectors that may be used to deliver a nucleic acid of the present invention are described below.

Adenoviral Vectors

[0146] A particular method for delivery of the nucleic acid involves the use of an adenovirus expression vector. Although adenovirus vectors are known to have a low capacity for integration into genomic DNA, this feature is counterbalanced by the high efficiency of gene transfer afforded by these vectors. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to ultimately express a tissue or cell specific construct that has been cloned therein. Knowledge of the genetic organization or adenovirus, a 36 kb, linear, double stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kb (Grunhaus and Horwitz, 1992).

AAV Vectors

[0147] The nucleic acid may be introduced into the cell using adenovirus assisted transfection. Increased transfection efficiencies have been reported in cell systems using adenovirus coupled systems (Kelleher and Vos, 1994; Cotten *et al.*, 1992; Curiel, 1994). Adeno associated virus (AAV) is an attractive vector system for use in the present invention as it has a high frequency of integration and it can infect nondividing cells, thus making it useful for delivery of genes into mammalian cells, for example, in tissue culture (Muzyczka, 1992) or *in vivo*. AAV has a broad host range for infectivity (Tratschin *et al.*, 1984; Laughlin *et al.*, 1986; Lebkowski *et al.*, 1988; McLaughlin *et al.*, 1988). Details concerning the generation and use of rAAV vectors are described in U.S. Patent Nos. 5,139,941 and 4,797,368, each incorporated herein by reference.

Retroviral Vectors

[0148] Retroviruses have promise as delivery vectors due to their ability to integrate their genes into the host genome, transferring a large amount of foreign genetic

material, infecting a broad spectrum of species and cell types and of being packaged in special cell lines (Miller, 1992).

[0149] In order to construct a retroviral vector, a nucleic acid (*e.g.*, one encoding a polynucleotide of interest) is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes but without the LTR and packaging components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into a special cell line (*e.g.*, by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

[0150] Lentiviruses are complex retroviruses, which, in addition to the common retroviral genes gag, pol, and env, contain other genes with regulatory or structural function. Lentiviral vectors are well known in the art (see, for example, Naldini *et al.*, 1996; Zufferey *et al.*, 1997; Blomer *et al.*, 1997; U.S. Pat. Nos. 6,013,516 and 5,994,136). Some examples of lentivirus include the Human Immunodeficiency Viruses: HIV-1, HIV-2 and the Simian Immunodeficiency Virus: SIV. Lentiviral vectors have been generated by multiply attenuating the HIV virulence genes, for example, the genes env, vif, vpr, vpu and nef are deleted making the vector biologically safe.

[0151] Recombinant lentiviral vectors are capable of infecting non-dividing cells and can be used for both *in vivo* and *ex vivo* gene transfer and expression of nucleic acid sequences. For example, recombinant lentivirus capable of infecting a non-dividing cell wherein a suitable host cell is transfected with two or more vectors carrying the packaging functions, namely gag, pol and env, as well as rev and tat is described in U.S. Pat. No. 5,994,136, incorporated herein by reference. One may target the recombinant virus by linkage of the envelope protein with an antibody or a particular ligand for targeting to a receptor of a particular cell-type. By inserting a sequence (including a regulatory region) of interest into the viral vector,

along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is now target-specific.

Other Viral Vectors

[0152] Other viral vectors may be employed as vaccine constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988), sindbis virus, cytomegalovirus and herpes simplex virus may be employed. They offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988; Horwich *et al.*, 1990).

Delivery Using Modified Viruses

[0153] A nucleic acid to be delivered may be housed within an infective virus that has been engineered to express a specific binding ligand. The virus particle will thus bind specifically to the cognate receptors of the target cell and deliver the contents to the cell. A novel approach designed to allow specific targeting of retrovirus vectors was developed based on the chemical modification of a retrovirus by the chemical addition of lactose residues to the viral envelope. This modification can permit the specific infection of hepatocytes *via* sialoglycoprotein receptors.

[0154] Another approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled *via* the biotin components by using streptavidin (Roux *et al.*, 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus *in vitro* (Roux *et al.*, 1989).

12. Vector Delivery and Cell Transformation

[0155] Suitable methods for nucleic acid delivery for transformation of an organelle, a cell, a tissue or an organism for use with the current invention are believed to include virtually any method by which a nucleic acid (*e.g.*, DNA) can be introduced into an organelle, a cell, a tissue or an organism, as described herein or as would be known to one of ordinary skill in the art. Such methods include, but are not limited to, direct delivery of DNA such as by *ex vivo* transfection (Wilson *et al.*, 1989, Nabel et al, 1989), by injection (U.S. Patent

Nos. 5,994,624, 5,981,274, 5,945,100, 5,780,448, 5,736,524, 5,702,932, 5,656,610, 5,589,466 and 5,580,859, each incorporated herein by reference), including microinjection (Harlan and Weintraub, 1985; U.S. Patent No. 5,789,215, incorporated herein by reference); by electroporation (U.S. Patent No. 5,384,253, incorporated herein by reference; Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984); by calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990); by using DEAE dextran followed by polyethylene glycol (Gopal, 1985); by direct sonic loading (Fechheimer *et al.*, 1987); by liposome mediated transfection (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Nicolau *et al.*, 1987; Wong *et al.*, 1980; Kaneda *et al.*, 1989; Kato *et al.*, 1991) and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988); by microprojectile bombardment (PCT Application Nos. WO 94/09699 and 95/06128; U.S. Patent Nos. 5,610,042; 5,322,783 5,563,055, 5,550,318, 5,538,877 and 5,538,880, and each incorporated herein by reference); by agitation with silicon carbide fibers (Kaeppler *et al.*, 1990; U.S. Patent Nos. 5,302,523 and 5,464,765, each incorporated herein by reference); by Agrobacterium mediated transformation (U.S. Patent Nos. 5,591,616 and 5,563,055, each incorporated herein by reference); by PEG mediated transformation of protoplasts (Omirulleh *et al.*, 1993; U.S. Patent Nos. 4,684,611 and 4,952,500, each incorporated herein by reference); by desiccation/inhibition mediated DNA uptake (Potrykus *et al.*, 1985), and any combination of such methods. Through the application of techniques such as these, organelle(s), cell(s), tissue(s) or organism(s) may be stably or transiently transformed.

13. *Ex vivo* Transformation

[0156] Methods for tranfecting vascular cells and tissues removed from an organism in an *ex vivo* setting are known to those of skill in the art. For example, cannine endothelial cells have been genetically altered by retrovial gene tranfer *in vitro* and transplanted into a canine (Wilson *et al.*, 1989). In another example, yucatan minipig endothelial cells were tranfected by retrovirus *in vitro* and transplated into an artery using a double-ballonw catheter (Nabel *et al.*, 1989). Thus, it is contemplated that cells or tissues may be removed and tranfected *ex vivo* using the nucleic acids of the present invention. In particular aspects, the transplanted cells or tissues may be placed into an organism. In preferred facets, a nucleic acid is expressed in the transplated cells or tissues.

14. Injection

[0157] In certain embodiments, a nucleic acid may be delivered to an organelle, a cell, a tissue or an organism *via* one or more injections (*i.e.*, a needle injection), such as, for example, subcutaneously, intradermally, intramuscularly, intervenously, intraperitoneally, etc. Methods of injection of vaccines are well known to those of ordinary skill in the art (*e.g.*, injection of a composition comprising a saline solution). Further embodiments of the present invention include the introduction of a nucleic acid by direct microinjection. Direct microinjection has been used to introduce nucleic acid constructs into Xenopus oocytes (Harland and Weintraub, 1985). The amount of agent used may vary upon the nature of the antigen as well as the organelle, cell, tissue or organism used

15. Electroporation

[0158] In certain embodiments of the present invention, a nucleic acid is introduced into an organelle, a cell, a tissue or an organism *via* electroporation. Electroporation involves the exposure of a suspension of cells and DNA to a high voltage electric discharge. In some variants of this method, certain cell wall degrading enzymes, such as pectin degrading enzymes, are employed to render the target recipient cells more susceptible to transformation by electroporation than untreated cells (U.S. Patent No. 5,384,253, incorporated herein by reference). Alternatively, recipient cells can be made more susceptible to transformation by mechanical wounding.

[0159] Transfection of eukaryotic cells using electroporation has been quite successful. Mouse pre B lymphocytes have been transfected with human kappa immunoglobulin genes (Potter *et al.*, 1984), and rat hepatocytes have been transfected with the chloramphenicol acetyltransferase gene (Tur Kaspa *et al.*, 1986) in this manner.

[0160] To effect transformation by electroporation in cells such as, for example, plant cells, one may employ either friable tissues, such as a suspension culture of cells or embryogenic callus or alternatively one may transform immature embryos or other organized tissue directly. In this technique, one would partially degrade the cell walls of the chosen cells by exposing them to pectin degrading enzymes (pectolyases) or mechanically wounding in a controlled manner. Examples of some species which have been transformed by electroporation

of intact cells include maize (U.S. Patent No. 5,384,253; Rhodes *et al.*, 1995; D'Halluin *et al.*, 1992), wheat (Zhou *et al.*, 1993), tomato (Hou and Lin, 1996), soybean (Christou *et al.*, 1987) and tobacco (Lee *et al.*, 1989).

[0161] One also may employ protoplasts for electroporation transformation of plant cells (Bates, 1994; Lazzeri, 1995). For example, the generation of transgenic soybean plants by electroporation of cotyledon derived protoplasts is described by Dhir and Widholm in International Patent Application No. WO 9217598, incorporated herein by reference. Other examples of species for which protoplast transformation has been described include barley (Lazerri, 1995), sorghum (Battraw *et al.*, 1991), maize (Bhattacharjee *et al.*, 1997), wheat (He *et al.*, 1994) and tomato (Tsukada, 1989).

16. Calcium Phosphate

[0162] In other embodiments of the present invention, a nucleic acid is introduced to the cells using calcium phosphate precipitation. Human KB cells have been transfected with adenovirus 5 DNA (Graham and Van Der Eb, 1973) using this technique. Also in this manner, mouse L(A9), mouse C127, CHO, CV 1, BHK, NIH3T3 and HeLa cells were transfected with a neomycin marker gene (Chen and Okayama, 1987), and rat hepatocytes were transfected with a variety of marker genes (Rippe *et al.*, 1990).

17. DEAE Dextran

[0163] In another embodiment, a nucleic acid is delivered into a cell using DEAE dextran followed by polyethylene glycol. In this manner, reporter plasmids were introduced into mouse myeloma and erythroleukemia cells (Gopal, 1985).

18. Sonication Loading

[0164] Additional embodiments of the present invention include the introduction of a nucleic acid by direct sonic loading. LTK fibroblasts have been transfected with the thymidine kinase gene by sonication loading (Fechheimer *et al.*, 1987).

19. Liposome Mediated Transfection

[0165] In a further embodiment of the invention, a nucleic acid may be entrapped in a lipid complex such as, for example, a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Also contemplated is an nucleic acid complexed with Lipofectamine (Gibco BRL) or Superfect (Qiagen).

[0166] Liposome mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Nicolau *et al.*, 1987). The feasibility of liposome mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells has also been demonstrated (Wong *et al.*, 1980).

[0167] In certain embodiments of the invention, a liposome may be complexed with a hemagglutinatin virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, a liposome may be complexed or employed in conjunction with nuclear non histone chromosomal proteins (HMG 1) (Kato *et al.*, 1991). In yet further embodiments, a liposome may be complexed or employed in conjunction with both HVJ and HMG 1. In other embodiments, a delivery vehicle may comprise a ligand and a liposome.

[0168] A unique liposomal delivery system has been successfully used in clinical trials. Liposomal gene delivery can be administered intravenously (iv) using multiple treatment cycles with reduced toxicity compared to intraperitoneal (ip) delivery. The major disadvantages of liposome delivery are transient transfection, lower transfection efficiency compared to viral vectors and potential to induce an inflammatory response (Ramesh *et al.*, 2001; Templeton *et al.*, 1997; Ruponen *et al.*, 2003). An exemplary liposomal delivery system uses 1,2-dioleoyl-3-trimethyl-ammoniopropane (DOTAP) and cholesterol (Ruponen *et al.*, 2003). This formulation combines with DNA to form bilamellar invaginated vesicles (liposomal BIVs) allowing delivery of RNA, DNA, siRNA, plasmids, viral particles and oligonucleotides. The structure of liposomal BIVs prolongs their plasma half-life by protecting them from harmful interaction with plasma

proteins and accumulation in non-target tissues and also inhibits DNA entry to the cell by endocytosis, which can lead to nucleic acid degradation (Simberg *et al.*, 2005). This liposomal delivery system has been used successfully in a clinical trial in which patients with end stage lung cancer were given 6 monthly iv infusions of liposomal fus 1 gene therapy without significant toxicity (Ito *et al.*, 2004).

20. Receptor-Mediated Transfection

[0169] Still further, a nucleic acid may be delivered to a target cell *via* receptor mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor mediated endocytosis that will be occurring in a target cell. In view of the cell type specific distribution of various receptors, this delivery method adds another degree of specificity to the present invention.

[0170] Certain receptor mediated gene targeting vehicles comprise a cell receptor specific ligand and a nucleic acid binding agent. Others comprise a cell receptor specific ligand to which the nucleic acid to be delivered has been operatively attached. Several ligands have been used for receptor mediated gene transfer (Wu and Wu, 1987; Wagner *et al.*, 1990; Perales *et al.*, 1994; Myers, EPO 0273085), which establishes the operability of the technique. Specific delivery in the context of another mammalian cell type has been described (Wu and Wu, 1993; incorporated herein by reference). In certain aspects of the present invention, a ligand will be chosen to correspond to a receptor specifically expressed on the target cell population.

[0171] In other embodiments, a nucleic acid delivery vehicle component of a cell specific nucleic acid targeting vehicle may comprise a specific binding ligand in combination with a liposome. The nucleic acid(s) to be delivered are housed within the liposome and the specific binding ligand is functionally incorporated into the liposome membrane. The liposome will thus specifically bind to the receptor(s) of a target cell and deliver the contents to a cell. Such systems have been shown to be functional using systems in which, for example, epidermal growth factor (EGF) is used in the receptor mediated delivery of a nucleic acid to cells that exhibit upregulation of the EGF receptor.

[0172] In still further embodiments, the nucleic acid delivery vehicle component of a targeted delivery vehicle may be a liposome itself, which will preferably comprise one or more

lipids or glycoproteins that direct cell specific binding. For example, lactosyl ceramide, a galactose terminal asialganglioside, have been incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes (Nicolau *et al.*, 1987). It is contemplated that the tissue specific transforming constructs of the present invention can be specifically delivered into a target cell in a similar manner.

21. Microprojectile Bombardment

[0173] Microprojectile bombardment techniques can be used to introduce a nucleic acid into at least one, organelle, cell, tissue or organism (U.S. Patent No. 5,550,318; U.S. Patent No. 5,538,880; U.S. Patent No. 5,610,042; and PCT Application WO 94/09699; each of which is incorporated herein by reference). This method depends on the ability to accelerate DNA coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). There are a wide variety of microprojectile bombardment techniques known in the art, many of which are applicable to the invention.

[0174] Microprojectile bombardment may be used to transform various cell(s), tissue(s) or organism(s), such as for example any plant species. Examples of species which have been transformed by microprojectile bombardment include monocot species such as maize (PCT Application WO 95/06128), barley (Ritala *et al.*, 1994; Hensgens *et al.*, 1993), wheat (U.S. Patent No. 5,563,055, incorporated herein by reference), rice (Hensgens *et al.*, 1993), oat (Torbet *et al.*, 1995; Torbet *et al.*, 1998), rye (Hensgens *et al.*, 1993), sugarcane (Bower *et al.*, 1992), and sorghum (Casas *et al.*, 1993; Hagio *et al.*, 1991); as well as a number of dicots including tobacco (Tomes *et al.*, 1990; Buising and Benbow, 1994), soybean (U.S. Patent No. 5,322,783, incorporated herein by reference), sunflower (Knittel *et al.* 1994), peanut (Singsit *et al.*, 1997), cotton (McCabe and Martinell, 1993), tomato (VanEck *et al.* 1995), and legumes in general (U.S. Patent No. 5,563,055, incorporated herein by reference).

[0175] In this microprojectile bombardment, one or more particles may be coated with at least one nucleic acid and delivered into cells by a propelling force. Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold particles or beads. Exemplary particles include those comprised of tungsten,

platinum and gold. It is contemplated that in some instances DNA precipitation onto metal particles would not be necessary for DNA delivery to a recipient cell using microprojectile bombardment. However, it is contemplated that particles may contain DNA rather than be coated with DNA. DNA coated particles may increase the level of DNA delivery *via* particle bombardment but are not, in and of themselves, necessary.

[0176] For the bombardment, cells in suspension are concentrated on filters or solid culture medium. Alternatively, immature embryos or other target cells may be arranged on solid culture medium. The cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate.

[0177] An illustrative embodiment of a method for delivering DNA into a cell (*e.g.*, a plant cell) by acceleration is the Biolistics Particle Delivery System, which can be used to propel particles coated with DNA or cells through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with cells, such as for example, a monocot plant cells cultured in suspension. The screen disperses the particles so that they are not delivered to the recipient cells in large aggregates. It is believed that a screen intervening between the projectile apparatus and the cells to be bombarded reduces the size of projectiles aggregate and may contribute to a higher frequency of transformation by reducing the damage inflicted on the recipient cells by projectiles that are too large.

22. Host Cells

[0178] As used herein, the terms "cell," "cell line," and "cell culture" may be used interchangeably. All of these terms also include their progeny, which is any and all subsequent generations. It is understood that all progeny may not be identical due to deliberate or inadvertent mutations. In the context of expressing a heterologous nucleic acid sequence, "host cell" refers to a prokaryotic or eukaryotic cell, and it includes any transformable organism that is capable of replicating a vector and/or expressing a heterologous gene encoded by a vector. A host cell can, and has been, used as a recipient for vectors. A host cell may be "transfected" or "transformed," which refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A transformed cell includes the primary subject cell and its progeny. As used herein, the terms "engineered" and "recombinant" cells or host cells are intended to refer to a cell into which an exogenous nucleic acid sequence, such as, for example, a

vector, has been introduced. Therefore, recombinant cells are distinguishable from naturally occurring cells which do not contain a recombinantly introduced nucleic acid.

[0179] In certain embodiments, it is contemplated that RNAs or proteinaceous sequences may be co expressed with other selected RNAs or proteinaceous sequences in the same host cell. Co-expression may be achieved by co transfecting the host cell with two or more distinct recombinant vectors. Alternatively, a single recombinant vector may be constructed to include multiple distinct coding regions for RNAs, which could then be expressed in host cells transfected with the single vector.

[0180] A tissue may comprise a host cell or cells to be transformed with a ZIP4 inhibitor or polynucleotide encoding same. The tissue may be part or separated from an organism. In certain embodiments, a tissue may comprise, but is not limited to, adipocytes, alveolar, ameloblasts, axon, basal cells, blood (*e.g.*, lymphocytes), blood vessel, bone, bone marrow, brain, breast, cartilage, cervix, colon, cornea, embryonic, endometrium, endothelial, epithelial, esophagus, facia, fibroblast, follicular, ganglion cells, glial cells, goblet cells, kidney, liver, lung, lymph node, muscle, neuron, ovaries, pancreas, peripheral blood, prostate, skin, skin, small intestine, spleen, stem cells, stomach, testes, and all cancers thereof.

[0181] In certain embodiments, the host cell or tissue may be comprised in at least one organism. In certain embodiments, the organism may be, but is not limited to, a prokayote (*e.g.*, a eubacteria, an archaea) or an eukaryote, as would be understood by one of ordinary skill in the art.

[0182] Numerous cell lines and cultures are available for use as a host cell, and they can be obtained through the American Type Culture Collection (ATCC), which is an organization that serves as an archive for living cultures and genetic materials (www.atcc.org). An appropriate host can be determined by one of skill in the art based on the vector backbone and the desired result. A plasmid or cosmid, for example, can be introduced into a prokaryote host cell for replication of many vectors. Cell types available for vector replication and/or expressioninclude, but are not limited to, bacteria, such as *E. coli* (*e.g.*, *E. coli* strain RR1, *E. coli* LE392, *E. coli* B, *E. coli* X 1776 (ATCC No. 31537) as well as *E. coli* W3110 (F, lambda, prototrophic, ATCC No. 273325), DH5α, JM109, and KC8, bacilli such as Bacillus subtilis; and other enterobacteriaceae such as Salmonella typhimurium, Serratia marcescens, various

Pseudomonas specie, as well as a number of commercially available bacterial hosts such as SURE® Competent Cells and SOLOPACK® Gold Cells (STRATAGENE®, La Jolla). In certain embodiments, bacterial cells such as *E. coli* LE392 are particularly contemplated as host cells for phage viruses.

[0183] Examples of eukaryotic host cells for replication and/or expression of a vector include, but are not limited to, HeLa, NIH3T3, Jurkat, 293, Cos, CHO, Saos, and PC12. Many host cells from various cell types and organisms are available and would be known to one of skill in the art. Similarly, a viral vector may be used in conjunction with either a eukaryotic or prokaryotic host cell, particularly one that is permissive for replication or expression of the vector.

[0184] Some vectors may employ control sequences that allow it to be replicated and/or expressed in both prokaryotic and eukaryotic cells. One of skill in the art would further understand the conditions under which to incubate all of the above described host cells to maintain them and to permit replication of a vector. Also understood and known are techniques and conditions that would allow large-scale production of vectors, as well as production of the nucleic acids encoded by vectors and their cognate polypeptides, proteins, or peptides.

23. Expression Systems

[0185] Numerous expression systems exist that comprise at least a part or all of the compositions discussed above. Prokaryote- and/or eukaryote-based systems can be employed for use with the present invention to produce nucleic acid sequences, or their cognate polypeptides, proteins and peptides. Many such systems are commercially and widely available.

[0186] The insect cell/baculovirus system can produce a high level of protein expression of a heterologous nucleic acid segment, such as described in U.S. Patent No. 5,871,986, 4,879,236, both herein incorporated by reference, and which can be bought, for example, under the name MAXBAC® 2.0 from INVITROGEN® and BACPACK™ BACULOVIRUS EXPRESSION SYSTEM FROM CLONTECH®.

[0187] Other examples of expression systems include STRATAGENE®'s COMPLETE CONTROL Inducible Mammalian Expression System, which involves a synthetic ecdysone-inducible receptor, or its pET Expression System, an *E. coli* expression system.

Another example of an inducible expression system is available from INVITROGEN®, which carries the T-REX™ (tetracycline-regulated expression) System, an inducible mammalian expression system that uses the full-length CMV promoter. INVITROGEN® also provides a yeast expression system called the Pichia methanolica Expression System, which is designed for high-level production of recombinant proteins in the methylotrophic yeast Pichia methanolica. One of skill in the art would know how to express a vector, such as an expression construct, to produce a nucleic acid sequence or its cognate polypeptide, protein, or peptide.

[0188] It is contemplated that the proteins, polypeptides or peptides produced by the methods of the invention may be "overexpressed", *i.e.*, expressed in increased levels relative to its natural expression in cells. Such overexpression may be assessed by a variety of methods, including radiolabeling and/or protein purification. However, simple and direct methods are preferred, for example, those involving SDS/PAGE and protein staining or western blotting, followed by quantitative analyses, such as densitometric scanning of the resultant gel or blot. A specific increase in the level of the recombinant protein, polypeptide or peptide in comparison to the level in natural cells is indicative of overexpression, as is a relative abundance of the specific protein, polypeptides or peptides in relation to the other proteins produced by the host cell and, *e.g.*, visible on a gel.

[0189] In some embodiments, the expressed proteinaceous sequence forms an inclusion body in the host cell, the host cells are lysed, for example, by disruption in a cell homogenizer, washed and/or centrifuged to separate the dense inclusion bodies and cell membranes from the soluble cell components. This centrifugation can be performed under conditions whereby the dense inclusion bodies are selectively enriched by incorporation of sugars, such as sucrose, into the buffer and centrifugation at a selective speed. Inclusion bodies may be solubilized in solutions containing high concentrations of urea (*e.g.* 8M) or chaotropic agents such as guanidine hydrochloride in the presence of reducing agents, such as β mercaptoethanol or DTT (dithiothreitol), and refolded into a more desirable conformation, as would be known to one of ordinary skill in the art.

V. SCREENING FOR MODULATORS OF THE PROTEIN FUNCTION

[0190] The present invention comprises methods for identifying modulators of the function of ZIP4. These assays may comprise random screening of large libraries of candidate substances; alternatively, the assays may be used to focus on particular classes of compounds

selected with an eye towards structural attributes that are believed to make them more likely to modulate the function of ZIP4. Alternatively, both assays may be employed concomitantly or in succession. By function, it is meant that one may assay for the ability to affect transcription, such as to affect RNAP for transcription, for example.

[0191] To identify a ZIP4 modulator, one generally will determine the function of ZIP4 in the presence and absence of the candidate substance, a modulator defined as any substance that alters function of ZIP4. For example, a method generally comprises:

[0192] (a) providing a candidate modulator;

[0193] (b) admixing the candidate modulator with an isolated compound or cell, or a suitable experimental animal;

[0194] (c) measuring one or more characteristics of the compound, cell or animal in step (c); and

[0195] (d) comparing the characteristic measured in step (c) with the characteristic of the compound, cell or animal in the absence of the candidate modulator,

[0196] wherein a difference between the measured characteristics indicates that the candidate modulator is, indeed, a modulator of the compound, cell or animal.

[0197] Assays may be conducted in cell free systems, in isolated cells, or in organisms including transgenic animals.

[0198] It will, of course, be understood that all of the screening methods of the present invention are useful in themselves notwithstanding the fact that effective candidates may not be found. The invention provides methods for screening for such candidates, not solely methods of finding them.

1. Modulators

[0199] As used herein the term "candidate substance" refers to any molecule that may potentially inhibit or, alternatively, enhance ZIP4 activity. The candidate substance may be a protein or fragment thereof, a small molecule, or even a nucleic acid molecule, for example, such as an siRNA. In some embodiments, a high throughput assay may be employed to identify modulators, such as is described in the Examples.

[0200] It may prove to be the case that the most useful pharmacological compounds will be compounds that are structurally related to other inhibitors of transcription factors. Using lead compounds to help develop improved compounds is know as "rational drug design" and includes not only comparisons with known inhibitors and activators, but predictions relating to the structure of target molecules.

[0201] The goal of rational drug design is to produce structural analogs of biologically active polypeptides or target compounds. By creating such analogs, it is possible to fashion drugs, which are more active or stable than the natural molecules, that have different susceptibility to alteration or that may affect the function of various other molecules. In a specific approach, one would generate a three-dimensional structure for a target molecule, or a fragment thereof. This could be accomplished by x-ray crystallography, computer modeling or by a combination of both approaches, for example.

[0202] It is also possible to use antibodies to ascertain the structure of a target compound activator or inhibitor. In principle, this approach yields a pharmacore upon which subsequent drug design can be based. It is possible to bypass protein crystallography altogether by generating anti-idiotypic antibodies to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of anti-idiotype would be expected to be an analog of the original antigen. The anti-idiotype could then be used to identify and isolate peptides from banks of chemically- or biologically-produced peptides. Selected peptides would then serve as the pharmacore. Anti-idiotypes may be generated using the methods described herein for producing antibodies, using an antibody as the antigen.

[0203] On the other hand, one may simply acquire, from various commercial sources, small molecule libraries that are believed to meet the basic criteria for useful drugs in an effort to "brute force" the identification of useful compounds. Screening of such libraries, including combinatorially-generated libraries (*e.g.*, peptide libraries), is a rapid and efficient way to screen large number of related (and unrelated) compounds for activity. Combinatorial approaches also lend themselves to rapid evolution of potential drugs by the creation of second, third and fourth generation compounds modeled of active, but otherwise undesirable compounds.

[0204] Candidate compounds may include fragments or parts of naturally-occurring compounds, or may be found as active combinations of known compounds, which are

otherwise inactive. It is proposed that compounds isolated from natural sources, such as animals, bacteria, fungi, plant sources, including leaves and bark, and marine samples may be assayed as candidates for the presence of potentially useful pharmaceutical agents. It will be understood that the pharmaceutical agents to be screened could also be derived or synthesized from chemical compositions or man-made compounds. Thus, it is understood that the candidate substance identified by the present invention may be peptide, polypeptide, polynucleotide, small molecule inhibitors or any other compounds that may be designed through rational drug design starting from known inhibitors or stimulators.

[0205] Other suitable modulators include antisense molecules, ribozymes, and antibodies (including single chain antibodies), each of which would be specific for the target molecule. For example, an antisense molecule that bound to a translational or transcriptional start site, or splice junctions, would be ideal candidate inhibitors.

[0206] In addition to the modulating compounds initially identified, the inventors also contemplate that other sterically similar compounds may be formulated to mimic the key portions of the structure of the modulators. Such compounds, which may include peptidomimetics of peptide modulators, may be used in the same manner as the initial modulators.

[0207] An inhibitor according to the present invention may be one which exerts its inhibitory or activating effect upstream, downstream or directly on ZIP4. Regardless of the type of inhibitor or activator identified by the present screening methods, the effect of the inhibition or activator by such a compound results in modulation of ZIP4, such as inhibition of the activity of ZIP4, as compared to that observed in the absence of the added candidate substance.

2. *In vitro* Assays

[0208] A quick, inexpensive and easy assay to run is an *in vitro* assay. Such assays generally use isolated molecules and can be run quickly and in large numbers, thereby increasing the amount of information obtainable in a short period of time. A variety of vessels may be used to run the assays, including test tubes, plates, dishes and other surfaces, such as dipsticks or beads, for example.

[0209] One example of a cell free assay is a binding assay. While not directly addressing function, the ability of a modulator to bind to a target molecule in a specific fashion is strong evidence of a related biological effect. For example, binding of a molecule to a target may, in and of itself, be inhibitory, due to steric, allosteric or charge-charge interactions. The target may be either free in solution, fixed to a support, expressed in or on the surface of a cell. Either the target or the compound may be labeled, thereby permitting determining of binding. Usually, the target will be the labeled species, decreasing the chance that the labeling will interfere with or enhance binding. Competitive binding formats can be performed in which one of the agents is labeled, and one may measure the amount of free label versus bound label to determine the effect on binding.

[0210] An exemplary technique for high throughput screening of compounds is described in WO 84/03564. Large numbers of small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. Bound polypeptide is detected by various methods.

3. *In cyto* Assays

[0211] The present invention also contemplates the screening of compounds for their ability to modulate ZIP4 in cells. Various cell lines can be utilized for such screening assays, including cells specifically engineered for this purpose.

[0212] Depending on the assay, culture may be required. The cell is examined using any of a number of different physiologic assays. Alternatively, molecular analysis may be performed, for example, looking at protein expression, mRNA expression (including differential display of whole cell or polyA RNA) and others.

4. *In vivo* Assays

[0213] *In vivo* assays involve the use of various animal models, including transgenic animals that have been engineered to have specific defects, or carry markers that can be used to measure the ability of a candidate substance to reach and effect different cells within the organism. Due to their size, ease of handling, and information on their physiology and genetic make-up, mice are a preferred embodiment, especially for transgenics. However, other

animals are suitable as well, including rats, rabbits, hamsters, guinea pigs, gerbils, woodchucks, cats, dogs, sheep, goats, pigs, cows, horses and monkeys (including chimps, gibbons and baboons). Assays for modulators may be conducted using an animal model derived from any of these species.

[0214] In such assays, one or more candidate substances are administered to an animal, and the ability of the candidate substance(s) to alter one or more characteristics, as compared to a similar animal not treated with the candidate substance(s), identifies a modulator. The characteristics may be any of those discussed above with regard to the function of a particular compound (*e.g.*, enzyme, receptor, hormone) or cell (*e.g.*, growth, tumorigenicity, survival), or instead a broader indication such as behavior, anemia, immune response, etc.

[0215] Treatment of these animals with test compounds will involve the administration of the compound, in an appropriate form, to the animal. Administration will be by any route that could be utilized for clinical or non-clinical purposes, including but not limited to oral, nasal, buccal, or even topical. Alternatively, administration may be by intratracheal instillation, bronchial instillation, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Specifically contemplated routes are systemic intravenous injection, regional administration *via* blood or lymph supply, or directly to an affected site.

[0216] Determining the effectiveness of a compound *in vivo* may involve a variety of different criteria. Also, measuring toxicity and dose response can be performed in animals in a more meaningful fashion than in *in vitro* or *in cyto* assays.

VI. PHARMACEUTICAL PREPARATIONS

[0217] Pharmaceutical compositions of the present invention comprise an effective amount of one or more agents that inhibit ZIP4 and may include one or more additional agents, wherein any of the agents are dissolved or dispersed in or provided with a pharmaceutically acceptable carrier. The phrases "pharmaceutical or pharmacologically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, such as, for example, a human, as appropriate. The preparation of an pharmaceutical composition that comprises at least one ZIP4 modulator (which for illustrative purposes will be referred to as an inhibitor) and, in some embodiments, an additional active ingredient, will be known to those of skill in the art in light of the present

disclosure, as exemplified by Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, incorporated herein by reference. Moreover, for animal (*e.g.*, human) administration, it will be understood that preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biological Standards.

[0218] As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, surfactants, antioxidants, preservatives (*e.g.*, antibacterial agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, drugs, drug stabilizers, gels, binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, such like materials and combinations thereof, as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, pp. 1289-1329, incorporated herein by reference). Except insofar as any conventional carrier is incompatible with the active ingredient, its use in the pharmaceutical compositions is contemplated.

[0219] The ZIP4 inhibitor may comprise different types of carriers depending on whether it is to be administered in solid, liquid or aerosol form, and whether it need to be sterile for such routes of administration as injection. The present invention can be administered intravenously, intradermally, transdermally, intrathecally, intraarterially, intraperitoneally, intranasally, intravaginally, intrarectally, topically, intramuscularly, subcutaneously, mucosally, orally, topically, locally, inhalation (*e.g.*, aerosol inhalation), injection, infusion, continuous infusion, localized perfusion bathing target cells directly, *via* a catheter, *via* a lavage, in cremes, in lipid compositions (*e.g.*, liposomes), or by other method or any combination of the forgoing as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, incorporated herein by reference).

[0220] The ZIP4 inhibitor may be formulated into a composition in a free base, neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts, *e.g.*, those formed with the free amino groups of a proteinaceous composition, or which are formed with inorganic acids such as for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric or mandelic acid. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as for example, sodium, potassium, ammonium, calcium or ferric hydroxides; or such organic bases as isopropylamine, trimethylamine, histidine or procaine. Upon formulation, solutions will be administered in a manner compatible with the

dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as formulated for parenteral administrations such as injectable solutions, or aerosols for delivery to the lungs, or formulated for alimentary administrations such as drug release capsules and the like.

[0221] Further in accordance with the present invention, the composition of the present invention suitable for administration is provided in a pharmaceutically acceptable carrier with or without an inert diluent. The carrier should be assimilable and includes liquid, semi-solid, *i.e.*, pastes, or solid carriers. Except insofar as any conventional media, agent, diluent or carrier is detrimental to the recipient or to the therapeutic effectiveness of a the composition contained therein, its use in administrable composition for use in practicing the methods of the present invention is appropriate. Examples of carriers or diluents include fats, oils, water, saline solutions, lipids, liposomes, resins, binders, fillers and the like, or combinations thereof. The composition may also comprise various antioxidants to retard oxidation of one or more component. Additionally, the prevention of the action of microorganisms can be brought about by preservatives such as various antibacterial and antifungal agents, including but not limited to parabens (*e.g.*, methylparabens, propylparabens), chlorobutanol, phenol, sorbic acid, thimerosal or combinations thereof.

[0222] In accordance with the present invention, the composition is combined with the carrier in any convenient and practical manner, *i.e.*, by solution, suspension, emulsification, admixture, encapsulation, absorption and the like. Such procedures are routine for those skilled in the art.

[0223] In a specific embodiment of the present invention, the composition is combined or mixed thoroughly with a semi-solid or solid carrier. The mixing can be carried out in any convenient manner such as grinding. Stabilizing agents can be also added in the mixing process in order to protect the composition from loss of therapeutic activity, *i.e.*, denaturation in the stomach. Examples of stabilizers for use in an the composition include buffers, amino acids such as glycine and lysine, carbohydrates such as dextrose, mannose, galactose, fructose, lactose, sucrose, maltose, sorbitol, mannitol, etc.

[0224] In further embodiments, the present invention may concern the use of a pharmaceutical lipid vehicle compositions that include the agent that inhibits ZIP4, one or more

lipids, and an aqueous solvent. As used herein, the term "lipid" will be defined to include any of a broad range of substances that is characteristically insoluble in water and extractable with an organic solvent. This broad class of compounds are well known to those of skill in the art, and as the term "lipid" is used herein, it is not limited to any particular structure. Examples include compounds which contain long-chain aliphatic hydrocarbons and their derivatives. A lipid may be naturally occurring or synthetic (*i.e.*, designed or produced by man). However, a lipid is usually a biological substance. Biological lipids are well known in the art, and include for example, neutral fats, phospholipids, phosphoglycerides, steroids, terpenes, lysolipids, glycosphingolipids, glycolipids, sulphatides, lipids with ether and ester-linked fatty acids and polymerizable lipids, and combinations thereof. Of course, compounds other than those specifically described herein that are understood by one of skill in the art as lipids are also encompassed by the compositions and methods of the present invention.

[0225] One of ordinary skill in the art would be familiar with the range of techniques that can be employed for dispersing a composition in a lipid vehicle. For example, the ZIP4 inhibitor may be dispersed in a solution containing a lipid, dissolved with a lipid, emulsified with a lipid, mixed with a lipid, combined with a lipid, covalently bonded to a lipid, contained as a suspension in a lipid, contained or complexed with a micelle or liposome, or otherwise associated with a lipid or lipid structure by any means known to those of ordinary skill in the art. The dispersion may or may not result in the formation of liposomes.

[0226] The actual dosage amount of a composition of the present invention administered to an animal patient can be determined by physical and physiological factors such as body weight, severity of condition, the type of disease being treated, previous or concurrent therapeutic interventions, idiopathy of the patient and on the route of administration. Depending upon the dosage and the route of administration, the number of administrations of a preferred dosage and/or an effective amount may vary according tot he response of the subject. The practitioner responsible for administration will, in any event, determine the concentration of active ingredient(s) in a composition and appropriate dose(s) for the individual subject.

[0227] In certain embodiments, pharmaceutical compositions may comprise, for example, at least about 0.1% of an active compound. In other embodiments, the an active compound may comprise between about 2% to about 75% of the weight of the unit, or between about 25% to about 60%, for example, and any range derivable therein. Naturally, the amount of

active compound(s) in each therapeutically useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

[0228] In other non-limiting examples, a dose may also comprise from about 1 microgram/kg/body weight, about 5 microgram/kg/body weight, about 10 microgram/kg/body weight, about 50 microgram/kg/body weight, about 100 microgram/kg/body weight, about 200 microgram/kg/body weight, about 350 microgram/kg/body weight, about 500 microgram/kg/body weight, about 1 milligram/kg/body weight, about 5 milligram/kg/body weight, about 10 milligram/kg/body weight, about 50 milligram/kg/body weight, about 100 milligram/kg/body weight, about 200 milligram/kg/body weight, about 350 milligram/kg/body weight, about 500 milligram/kg/body weight, to about 1000 mg/kg/body weight or more per administration, and any range derivable therein. In non-limiting examples of a derivable range from the numbers listed herein, a range of about 5 mg/kg/body weight to about 100 mg/kg/body weight, about 5 microgram/kg/body weight to about 500 milligram/kg/body weight, etc., can be administered, based on the numbers described above.

1. Alimentary Compositions and Formulations

[0229] In preferred embodiments of the present invention, the ZIP4 inhibitor is formulated to be administered *via* an alimentary route. Alimentary routes include all possible routes of administration in which the composition is in direct contact with the alimentary tract. Specifically, the pharmaceutical compositions disclosed herein may be administered orally, buccally, rectally, or sublingually. As such, these compositions may be formulated with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard- or soft- shell gelatin capsule, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet.

[0230] In certain embodiments, the active compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like (Mathiowitz *et al.*, 1997; Hwang *et al.*, 1998; U.S. Pat. Nos. 5,641,515; 5,580,579 and 5,792, 451, each specifically incorporated herein by reference in

its entirety). The tablets, troches, pills, capsules and the like may also contain the following: a binder, such as, for example, gum tragacanth, acacia, cornstarch, gelatin or combinations thereof; an excipient, such as, for example, dicalcium phosphate, mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate or combinations thereof; a disintegrating agent, such as, for example, corn starch, potato starch, alginic acid or combinations thereof; a lubricant, such as, for example, magnesium stearate; a sweetening agent, such as, for example, sucrose, lactose, saccharin or combinations thereof; a flavoring agent, such as, for example peppermint, oil of wintergreen, cherry flavoring, orange flavoring, etc. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar, or both. When the dosage form is a capsule, it may contain, in addition to materials of the above type, carriers such as a liquid carrier. Gelatin capsules, tablets, or pills may be enterically coated. Enteric coatings prevent denaturation of the composition in the stomach or upper bowel where the pH is acidic. See, *e.g.*, U.S. Pat. No. 5,629,001. Upon reaching the small intestines, the basic pH therein dissolves the coating and permits the composition to be released and absorbed by specialized cells, *e.g.*, epithelial enterocytes and Peyer's patch M cells. A syrup of elixir may contain the active compound sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations.

[0231] For oral administration the compositions of the present invention may alternatively be incorporated with one or more excipients in the form of a mouthwash, dentifrice, buccal tablet, oral spray, or sublingual orally- administered formulation. For example, a mouthwash may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution). Alternatively, the active ingredient may be incorporated into an oral solution such as one containing sodium borate, glycerin and potassium bicarbonate, or dispersed in a dentifrice, or added in a therapeutically-effective amount to a composition that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants. Alternatively the compositions may be fashioned into a tablet or solution form that may be placed under the tongue or otherwise dissolved in the mouth.

[0232] Additional formulations which are suitable for other modes of alimentary administration include suppositories. Suppositories are solid dosage forms of various weights and shapes, usually medicated, for insertion into the rectum. After insertion, suppositories soften, melt or dissolve in the cavity fluids. In general, for suppositories, traditional carriers may include, for example, polyalkylene glycols, triglycerides or combinations thereof. In certain embodiments, suppositories may be formed from mixtures containing, for example, the active ingredient in the range of about 0.5% to about 10%, and preferably about 1% to about 2%.

2. Parenteral Compositions and Formulations

[0233] In further embodiments, ZIP4 inhibitor may be administered *via* a parenteral route. As used herein, the term "parenteral" includes routes that bypass the alimentary tract. Specifically, the pharmaceutical compositions disclosed herein may be administered for example, but not limited to intravenously, intradermally, intramuscularly, intraarterially, intrathecally, subcutaneous, or intraperitoneally U.S. Pat. Nos. 6,7537,514, 6,613,308, 5,466,468, 5,543,158; 5,641,515; and 5,399,363 (each specifically incorporated herein by reference in its entirety)..

[0234] Solutions of the active compounds as free base or pharmacologically acceptable salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (U.S. Patent 5,466,468, specifically incorporated herein by reference in its entirety). In all cases the form must be sterile and must be fluid to the extent that easy injectability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*i.e.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various

antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

[0235] For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous, and intraperitoneal administration. In this connection, sterile aqueous media that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

[0236] Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. A powdered composition is combined with a liquid carrier such as, *e.g.*, water or a saline solution, with or without a stabilizing agent.

3. Miscellaneous Pharmaceutical Compositions and Formulations

[0237] In other preferred embodiments of the invention, the active compound ZIP4 inhibitor may be formulated for administration *via* various miscellaneous routes, for example, topical (*i.e.*, transdermal) administration, mucosal administration (intranasal, vaginal, etc.) and/or inhalation.

[0238] Pharmaceutical compositions for topical administration may include the active compound formulated for a medicated application such as an ointment, paste, cream or powder. Ointments include all oleaginous, adsorption, emulsion and water-solubly based compositions for topical application, while creams and lotions are those compositions that include an emulsion base only. Topically administered medications may contain a penetration enhancer to facilitate adsorption of the active ingredients through the skin. Suitable penetration enhancers include glycerin, alcohols, alkyl methyl sulfoxides, pyrrolidones and luarocapram. Possible bases for compositions for topical application include polyethylene glycol, lanolin, cold cream and petrolatum as well as any other suitable absorption, emulsion or water-soluble ointment base. Topical preparations may also include emulsifiers, gelling agents, and antimicrobial preservatives as necessary to preserve the active ingredient and provide for a homogenous mixture. Transdermal administration of the present invention may also comprise the use of a "patch". For example, the patch may supply one or more active substances at a predetermined rate and in a continuous manner over a fixed period of time.

[0239] In certain embodiments, the pharmaceutical compositions may be delivered by eye drops, intranasal sprays, inhalation, and/or other aerosol delivery vehicles. Methods for delivering compositions directly to the lungs *via* nasal aerosol sprays has been described *e.g.*, in U.S. Pat. Nos. 5,756,353 and 5,804,212 (each specifically incorporated herein by reference in its entirety). Likewise, the delivery of drugs using intranasal microparticle resins (Takenaga *et al.*, 1998) and lysophosphatidyl-glycerol compounds (U.S. Pat. No. 5,725, 871, specifically incorporated herein by reference in its entirety) are also well-known in the pharmaceutical arts. Likewise, transmucosal drug delivery in the form of a polytetrafluoroetheylene support matrix is described in U.S. Pat. No. 5,780,045 (specifically incorporated herein by reference in its entirety).

[0240] The term aerosol refers to a colloidal system of finely divided solid of liquid particles dispersed in a liquefied or pressurized gas propellant. The typical aerosol of the present invention for inhalation will consist of a suspension of active ingredients in liquid propellant or a mixture of liquid propellant and a suitable solvent. Suitable propellants include hydrocarbons and hydrocarbon ethers. Suitable containers will vary according to the pressure requirements of the propellant. Administration of the aerosol will vary according to subject's age, weight and the severity and response of the symptoms.

VII. COMBINATION TREATMENTS

[0241] In certain aspects, the therapy of the invention may be combined with other agents that are effective in the treatment of hyperproliferative disease, such as anti-cancer agents. An "anti-cancer" agent is capable of negatively affecting cancer in a subject, for example, by killing cancer cells, inducing apoptosis in cancer cells, reducing the growth rate of cancer cells, reducing the incidence or number of metastases, reducing tumor size, inhibiting tumor growth, reducing the blood supply to a tumor or cancer cells, promoting an immune response against cancer cells or a tumor, preventing or inhibiting the progression of cancer, or increasing the lifespan of a subject with cancer. The compositions of the present invention are considered anti-cancer agents.

[0242] More generally, these other compositions or methods would be provided in a combined amount effective to kill or inhibit proliferation of the cancer cell. This process may involve contacting the cells with the ZIP4 inhibitor, which may be referred to as the first agent, and the second agent(s) or multiple factor(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the expression construct and the other includes the second agent(s).

[0243] Tumor cell resistance to chemotherapy and radiotherapy agents represents a major problem in clinical oncology. One goal of current cancer research is to find ways to improve the efficacy of chemo- and radiotherapy by combining it with gene therapy. For example, the herpes simplex-thymidine kinase (HS-tK) gene, when delivered to brain tumors by a retroviral vector system, successfully induced susceptibility to the antiviral agent ganciclovir (Culver, *et al.*, 1992). In the context of the present invention, it is contemplated that the ZIP4

inhibitor therapy could be used similarly in conjunction with chemotherapeutic, radiotherapeutic, or immunotherapeutic intervention, in addition to other pro-apoptotic or cell cycle regulating agents, for example.

[0244] Alternatively, the inventive therapy may precede or follow the other agent treatment by intervals ranging from minutes to weeks. In embodiments where the other agent and expression construct are applied separately to the cell, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the agent and expression construct would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one may contact the cell with both modalities within about 12-24 h of each other and, more preferably, within about 6-12 h of each other. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several d (2, 3, 4, 5, 6 or 7) to several wk (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

[0245] Various combinations may be employed, wherein inventive therapy is "A" and the secondary agent, such as radio- or chemotherapy, is "B":

[0246] A/B/A B/A/B B/B/A A/A/B A/B/B B/A/A A/B/B/B B/A/B/B

[0247] B/B/B/A B/B/A/B A/A/B/B A/B/A/B A/B/B/A B/B/A/A

[0248] B/A/B/A B/A/A/B A/A/A/B B/A/A/A A/B/A/A A/A/B/A

[0249] Administration of the therapeutic agents of the present invention to a patient will follow general protocols for the administration of chemotherapeutics, taking into account the toxicity, if any, of the composition. It is expected that the treatment cycles would be repeated as necessary. It also is contemplated that various standard therapies, as well as surgical intervention, may be applied in combination with the described hyperproliferative cell therapy.

1. Chemotherapy

[0250] Cancer therapies also include a variety of combination therapies with both chemical and radiation based treatments. Combination chemotherapies include, for example, cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, busulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide (VP16), tamoxifen,

raloxifene, estrogen receptor binding agents, oxaliplatin, taxol, gemcitabien, navelbine, farnesyl-protein tansferase inhibitors, transplatinum, 5-fluorouracil, vincristin, vinblastin and methotrexate, or any analog or derivative variant of the foregoing. Any of these therapies may be used in combination with the invention.

[0251] There is no effective specific chemotherapeutics for pancreatic cancer. The way the chemotherapy is given depends on factors such as the type and stage of the cancer being treated, and one of skill in the art would identify which chemotherapy to use given the type and stage of the cancer, for example. Systemic chemotherapy with single-agent gemcitabine or a gemcitabine-based regimen still remains one of the standards of care for the treatment of patients with locally advanced and metastatic pancreatic cancer. A recent report showed that addition of Gemcitabine to radiation and 5-FU treatment after the surgery helped patient live longer. For advanced, inoperable pancreatic cancer, patients with combination of Gemcitabine and Cisplatin or oxaliplatin (Eloxatin) treatment survive longer than single drug treatment.

2. Radiotherapy

[0252] Other factors that cause DNA damage and have been used extensively include what are commonly known as γ-rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves and UV-irradiation. It is most likely that all of these factors effect a broad range of damage on DNA, on the precursors of DNA, on the replication and repair of DNA, and on the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 wk), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

[0253] The terms "contacted" and "exposed," when applied to a cell, are used herein to describe the process by which a therapeutic construct and a chemotherapeutic or radiotherapeutic agent are delivered to a target cell or are placed in direct juxtaposition with the target cell. To achieve cell killing or stasis, both agents are delivered to a cell in a combined amount effective to kill the cell or prevent it from dividing.

3. Immunotherapy

[0254] Immunotherapeutics, generally, rely on the use of immune effector cells and molecules to target and destroy cancer cells. The immune effector may be, for example, an antibody specific for some marker on the surface of a tumor cell. The antibody alone may serve as an effector of therapy or it may recruit other cells to actually effect cell killing. The antibody also may be conjugated to a drug or toxin (chemotherapeutic, radionuclide, ricin A chain, cholera toxin, pertussis toxin, etc.) and serve merely as a targeting agent. Alternatively, the effector may be a lymphocyte carrying a surface molecule that interacts, either directly or indirectly, with a tumor cell target. Various effector cells include cytotoxic T cells and NK cells.

[0255] Immunotherapy, thus, could be used as part of a combined therapy, in conjunction with the inventive therapy. The general approach for combined therapy is discussed below. Generally, the tumor cell must bear some marker that is amenable to targeting, *i.e.*, is not present on the majority of other cells. Many tumor markers exist and any of these may be suitable for targeting in the context of the present invention. Common tumor markers include carcinoembryonic antigen, prostate specific antigen, urinary tumor associated antigen, fetal antigen, tyrosinase (p97), gp68, TAG-72, HMFG, Sialyl Lewis Antigen, MucA, MucB, PLAP, estrogen receptor, laminin receptor, erb B and p155.

4. Genes

[0256] In yet another embodiment, the secondary treatment is a gene therapy in which a therapeutic polynucleotide is administered before, after, or at the same time as the inventive ZIP4 inhibitor. A variety of proteins are encompassed within the invention, some of which include inducers of apoposis and/or inhibitors of cell proliferation, for example.

5. Surgery

[0257] Approximately 60% of persons with cancer will undergo surgery of some type, which includes preventative, diagnostic or staging, curative and palliative surgery. Curative surgery is a cancer treatment that may be used in conjunction with other therapies, such as the treatment of the present invention, chemotherapy, radiotherapy, hormonal therapy, gene therapy, immunotherapy and/or alternative therapies.

[0258] Curative surgery includes resection in which all or part of cancerous tissue is physically removed, excised, and/or destroyed. Tumor resection refers to physical removal of at least part of a tumor. In addition to tumor resection, treatment by surgery includes laser surgery, cryosurgery, electrosurgery, and miscopically controlled surgery (Mohs' surgery). It is further contemplated that the present invention may be used in conjunction with removal of superficial cancers, precancers, or incidental amounts of normal tissue.

[0259] Upon excision of part of all of cancerous cells, tissue, or tumor, a cavity may be formed in the body. Treatment may be accomplished by perfusion, direct injection or local application of the area with an additional anti-cancer therapy. Such treatment may be repeated, for example, every 1, 2, 3, 4, 5, 6, or 7 days, or every 1, 2, 3, 4, and 5 weeks or every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months. These treatments may be of varying dosages as well.

6. Other agents

[0260] It is contemplated that other agents may be used in combination with the present invention to improve the therapeutic efficacy of treatment. These additional agents include immunomodulatory agents, agents that affect the upregulation of cell surface receptors and GAP junctions, cytostatic and differentiation agents, inhibitors of cell adhesion, or agents that increase the sensitivity of the hyperproliferative cells to apoptotic inducers. Immunomodulatory agents include tumor necrosis factor; interferon alpha, beta, and gamma; IL-2 and other cytokines; F42K and other cytokine analogs; or MIP-1, MIP-1beta, MCP-1, RANTES, and other chemokines. It is further contemplated that the upregulation of cell surface receptors or their ligands such as Fas / Fas ligand, DR4 or DR5 / TRAIL would potentiate the apoptotic inducing abililties of the present invention by establishment of an autocrine or paracrine effect on hyperproliferative cells. Increases intercellular signaling by elevating the number of GAP junctions would increase the anti-hyperproliferative effects on the neighboring hyperproliferative cell population. In other embodiments, cytostatic or differentiation agents can be used in combination with the present invention to improve the anti-hyerproliferative efficacy of the treatments. Inhibitors of cell adehesion are contemplated to improve the efficacy of the present invention. Examples of cell adhesion inhibitors are focal adhesion kinase (FAKs) inhibitors and Lovastatin. It is further contemplated that other agents that increase the sensitivity of a hyperproliferative cell to apoptosis, such as the antibody c225, could be used in combination with the present invention to improve the treatment efficacy.

[0261] Hormonal therapy may also be used in conjunction with the present invention or in combination with any other cancer therapy previously described. The use of hormones may be employed in the treatment of certain cancers such as breast, prostate, ovarian, or cervical cancer to lower the level or block the effects of certain hormones such as testosterone or estrogen. This treatment is often used in combination with at least one other cancer therapy as a treatment option or to reduce the risk of metastases.

VIII. KITS OF THE INVENTION

[0262] Any of the compositions described herein may be comprised in a kit. In a non-limiting example, an agent that inhibits ZIP4 and an additional agent, including an additional anti-cancer agent, in specific embodiments, may be comprised in a kit. The kits will thus comprise its contents in suitable container means.

[0263] The kits may comprise a suitably aliquoted agent that inhibits ZIP4, a pharmaceutical carrier, such as a lipid, and including a liposome, and/or an additional agent. Compositions of the present invention, whether labeled or unlabeled, as may be used to prepare a standard curve for a detection assay. The components of the kits may be packaged either in aqueous media or in lyophilized form. The container means of the kits will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which a component may be placed, and preferably, suitably aliquoted. Where there are more than one component in the kit, the kit also will generally contain a second, third or other additional container into which the additional components may be separately placed. However, various combinations of components may be comprised in a vial. The kits of the present invention also will typically include a means for containing the ZIP4 inhibitor, lipid, additional agent, and any other reagent containers in close confinement for commercial sale. Such containers may include injection or blow molded plastic containers into which the desired vials are retained.

[0264] Therapeutic kits of the present invention are kits that may comprise an agent that inhibits ZIP4, such as siRNA molecules that are directed to ZIP4. Additional agents may include chemical compounds or pharmaceutically acceptable salts thereof, a protein, polypeptide, peptide, inhibitor, gene, polynucleotide, vector and/or other effector. Such kits may generally contain the compositions in a pharmaceutically acceptable formulation. The kit may have a single container means, and/or it may have distinct container means for each compound.

[0265] When the components of the kit are provided in one and/or more liquid solutions, the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly preferred. The agent may also be formulated into a syringeable composition. In this case, the container means may itself be a syringe, pipette, and/or other such like apparatus, from which the formulation may be applied to an affected area of the body, injected into an animal, and/or even applied to and/or mixed with the other components of the kit. The formulation may be suitable for systemic or local delivery.

[0266] In some embodiments, the components of the kit may be provided as dried powder(s). When reagents and/or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container means and may be sterile.

[0267] The container means will generally include at least one vial, test tube, flask, bottle, syringe and/or other container means, into which the inhibitory formulation is placed, and preferably is suitably allocated. The kits may also comprise a second container means for containing a sterile, pharmaceutically acceptable buffer and/or other diluent.

[0268] The kits of the present invention will also typically include a means for containing the vials in close confinement for commercial sale, such as, *e.g.*, injection and/or blow-molded plastic containers into which the desired vials are retained.

[0269] Irrespective of the number and/or type of containers, the kits of the invention may also comprise, and/or be packaged with, an instrument for assisting with the injection/administration and/or placement of the ultimate composition within or to the body of an animal. Such an instrument may be a syringe, pipette, forceps, and/or any such medically approved delivery vehicle.

[0270] In specific embodiments, the kit comprises an additional composition for treatment of cancer, including a chemotherapeutic drug. The kit may be tailored to include chemotherapeutic drugs suitable for the type of cancer being treated. For example, kits may be formulated for individuals with breast cancer and may include in addition to the ZIP4 inhibitor one or more breast cancer drugs, such as Taxol, herceptin, tamoxifen, paclitaxel, gemcitabine, and so forth.

EXAMPLES

[0271] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

EXEMPLARY MATERIALS AND METHODS FOR EXAMPLES 2-5

[0272] *Cells, Chemicals, and Human Tissue Specimens.* Human pancreatic cancer cell lines, Panc-1, MIA PaCa-2, BxPC-3, Hs766T, ASPC-1, Capan-1, HPAF-II, and PL45, were purchased from the American Type Culture Collection (ATCC). The HPDE cells were also provided from an individual laboratory (Ontario Cancer Institute, Toronto, ON, Canada) (Furukawa *et al.*, 1996; Ouyang *et al.*, 2000). All cells were cultured as described (Li *et al.*, 2004; Li *et al.*, 2006). The human ZIP4 (hZIP4) antibody was generated in rabbits against a KLH-conjugated 14-aa synthetic peptide and affinity-purified basically as described (Liuzzi *et al.*, 2004). Other chemicals were from Sigma. Human pancreatic adenocarcinoma specimens were collected from patients who underwent surgery according to an approved human protocol.

[0273] *ZIP4 mRNA Detection.* Sample preparation and microarray analysis (Affymetrix) were previously described (Logsdon *et al.*, 2003). The ZIP4 mRNA was analyzed by real-time RT-PCR as described (Li *et al.*, 2004; Li *et al.*, 2006). Briefly, real-time PCR was performed with total RNA by using the SYBR supermix kit (Bio-Rad). PCR included the following components: 100 nM each primer, diluted cDNA templates and iQ SYBR green supermix, and running for 40 cycles at 95°C for 20 sec and 60°C for 1 min. PCR efficiency was examined by serially diluting the template cDNA, and the melting-curve data were collected to check PCR specificity. Each cDNA sample was run as triplicates, and the corresponding no-reverse transcriptase (RT) mRNA sample was included as a negative control. The β-actin primer was included in every plate to avoid sample variations. The mRNA level of each sample for each

gene was normalized to that of the β-actin mRNA. The amount of PCR products was measured by threshold cycle (Ct) values. The relative mRNA level was presented as unit values of $2^{[Ct_{(\beta\text{-actin})} - Ct_{(\text{gene of interest})}]}$. The primer sequences for human ZIP4 gene (SLC39A4) are: sense 5'-ATGTCAGGAGCGGGTCTTGC -3' (SEQ ID NO: 5); and antisense 5'-GCTGCTGTGCTGCTGGAAC-3' (SEQ ID NO:6).

[0274] *Immunohistochemical Staining.* Human pancreatic adenocarcinoma and surrounding normal tissues were collected and processed into 5-μm slices. Fixed tissue slides were incubated in 0.3% hydrogen peroxide solution to quench endogenous peroxidase activity for 15 min and were subsequently washed with PBS. The slides were then incubated in blocking buffer for 30 min at room temperature before adding anti-hZIP4 antibody and incubated for 60 min at room temperature. The rabbit polyclonal anti-hZIP4 antibody was prepared basically as described (Liuzzi *et al.*, 2004). After washing with PBS, the section was incubated with biotinylated secondary antibody for 30 min. An avidin–biotin reaction using peroxidase enzyme was used for protein detection (ABC kit; Vector Laboratories). Immune complexes were detected with diaminobenzidine (DAB) under a phase-contrast microscope. Mouse s.c. tumors were collected and processed into 5-μm slices. Fixed tissue slides were incubated with anti-Ki67 antibody (Biodesign International) for 30 min at 4°C, before DAB visualization, and the sections were then mounted and observed under a phase-contrast microscope.

[0275] *Stable Cell Line Selection.* ZIP4 overexpression cells were selected in MIA PaCa-2 cells with retrovirus vector pBabe (Clontech), following manufacturer's instructions. Briefly, full-length human ZIP4 cDNA (NM_130849) (SEQ ID NO: 1) was cloned into pBabe vector, and the recombinant plasmid was cotransfected into 293T cells with plasmids PegPam3 and RDF (containing RD114 envelope). Viral supernatants were collected and transduced to the target cells. Stable cell lines expressing ZIP4 (MIA-ZIP4) or empty vector (MIA-V) were selected by adding 0.5 μg/ml puromycin into the medium. Three individual lines were selected for each stable cell.

[0276] *Western Blot Analysis.* MIA-V and MIA-ZIP4 cells were lysed with ice-cold lysis buffer (20 mM Tris_HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and protease inhibitor mixture) for 30 min on ice. Cell lysates were then collected after centrifugation at 13,000 x *g* for 5 min at 4°C. Sixty micrograms of lysate protein was

loaded, and total cellular protein was separated with 15% SDS/PAGE and then transblotted overnight at 4°C onto Hybond-P PVDF membrane (Amersham Biosciences). The membrane was probed with anti- ZIP4 (1:500) or anti- β -actin (1:3,000) antibody at room temperature for 1 h and then washed three times with 0.1% Tween 20-TBS and incubated in a horseradish peroxidase-linked secondary antibody (1:2,000) for 1 h at room temperature. The membrane was washed three times with 0.1% Tween 20-TBS, and the immunoreactive bands were detected by using ECL plus reagent kit.

[0277] *Cell Proliferation Assay.* Cell proliferation was analyzed with the MTS assay. Stable MIA PaCa-2 cells were seeded in 96-well plates ($2x10^3$ cells per well), and serum-starved (0% FBS) for 24 h. Cell growth was assessed 1, 2, 3, and 5 days after starvation. For zinc-dependent assay, MIA-V and MIA-ZIP4 cells were treated with a membrane-permeable metal chelator, *N,N,N',N*-tetrakis(2- pyridylmethyl) ethylenediamine (TPEN; Sigma) at 4 μM for 1 h at 37°C. Cells were washed with PBS to remove excess TPEN because TPEN is toxic to cells with long-time incubation. Cells were then incubated with DMEM in the presence of 0, 1, 5, 25, 50, and 125 μM $ZnCl_2$. Cell growth was assessed 1 day after treatment. Twenty microliters of MTS reagent mixed with 100 μl of growth medium was added to each well and incubated at 37°C for 2 h. Absorbance was recorded at 490 nm with an EL-800 universal microplate reader (Bio-Tek Instruments).

[0278] *Pancreatic Cancer Mouse Models.* Subconfluent MIA-ZIP4 or MIA-V cells were harvested by trypsinization and resuspended in DMEM. Only single-cell suspensions with >95% viability were used. The cells (3×10^6) were inoculated either into the right flank (s.c. tumor model) or the body of the pancreas (orthotopic tumor model) of 5- to 6-week-old male nude mice (NCI-Charles River). For the s.c. tumor model, the tumor size was measured weekly by using a digital caliper (VWR International), and the tumor volume was determined with the formula: tumor volume [mm^3]=(length [mm])x(width [mm])^2 x 0.52. For intrapancreatic injections, mice were anesthetized with 2.5% avertin, and a 0.5- to 1-cm incision was made in the left subcostal region. The tumor cells (3×10^6) in a volume of 50 μl were injected into the body of the pancreas. The peritoneum and skin were closed with a 4.0 surgical suture. After 4 weeks, all surviving mice were euthanized by an overdose of CO_2 exposure and evaluated macroscopically for the presence of orthotopic tumors and the metastases in the abdominal cavity. The orthotopic and metastatic tumor nodules were then explanted, counted, and

measured. For both s.c. and orthotopic experiments, the animals were euthanized when their tumor size reached 2 cm in diameter or the animals became moribund during the observation period, and the time of euthanization was recorded as the time of mortality.

[0279] *Zinc Transport and Concentration.* For cell lines, MIA-V and MIAZIP4 cells were seeded in 6-well plates and treated with TPEN at 4 μM for 1 h at 37°C. Cells were washed with PBS to remove excessive TPEN. Cells were then incubated with DMEM in the presence of 10μM $ZnCl_2$ for 5 min before collection. The cells were washed twice in PBS buffer. Cell pellets (~10^4 cells in each sample) were digested in 70% (vol/vol) HNO_3 at 70°C for 1 h. For tissues, s.c. or orthotopic primary tumors of the same size from MIA-V- or MIA-ZIP4-injected mice were collected and homogenized. Homogenized mouse tissues (~50 mg) were placed in septum-sealed glass tubes and treated with 0.1 ml of 70% (vol/vol) HNO_3 overnight. All these samples were finally diluted with HPLC-grade water to 2ml for quantitative assay. Zinc concentrations in cell lines and tissues were determined by inductively coupled plasma mass spectrometry (ICPMS) (ELAN 9000, PerkinElmer) and normalized to the total protein content.

[0280] *Statistical Analysis.* Quantitative results are shown as means ± standard deviations. The statistical analysis was performed by Student's *t*-test for paired data between control and treated groups or one-way ANOVA for data from multiple groups. *P* values <0.05 were considered significant.

EXAMPLE 2

ZIP4 IS OVEREXPRESSED IN HUMAN PANCREATIC CANCER TISSUE SPECIMENS AND CELL LINES

[0281] ZIP4 (SLC39A4) mRNA (SEQ ID NO:1) was found by microarray to be overexpressed in human pancreatic cancer tissue samples compared with normal and pancreatitis samples (FIG. 1A). To confirm the microarray data, ZIP4 expression was examined in 17 pairs of human pancreatic cancer tissues with the surrounding normal tissues and five pancreatitis tissues. ZIP4 expression was also examined in eight human pancreatic cancer cell lines. ZIP4 mRNA was substantially overexpressed in 16 of 17 (94%) clinical pancreatic- adenocarcinoma samples compared with that in their surrounding normal tissues (FIG. 1B and Table 1). In all five pancreatitis tissues, ZIP4 mRNA was as low as that in the surrounding normal tissues. Overall,

the average mRNA expression in 17 pancreatic cancer tissues was 5.5 times that in the surrounding normal tissues. The tumor samples also showed strong immunoreactivity to human ZIP4 Ab (FIG. 1C). ZIP4 expression was differentially higher in seven human pancreatic cancer cell lines (Panc-1, BxPC-3, Hs766T, ASPC-1, Capan-1, HPAF-II, and PL45) compared with that in human pancreatic ductal epithelium (HPDE) cells, but the expression of ZIP4 in MIA PaCa-2 cells was similar to that in HPDE cells (FIG. 1D). Thus, high expression of ZIP4 in the majority of pancreatic cancer tissue specimens and cell lines shows that this zinc transporter contributes to cancer growth.

TABLE 1. HUMAN ZIP4 MRNA LEVELS IN 17 PAIRS OF HUMAN ADENOCARCINOMA TISSUES

PATIENT NO.	MRNA* (NORMAL TISSUES)	MRNA* (TUMOR TISSUES)	FOLD INCREASE (TUMOR/NORMAL)
IX.	2.5E-05	1.4E-03	53.6
X.	2.2E-04	8.2E-03	38.2
XI.	7.8E-05	1.6E-03	20.4
XII.	5.5E-05	7.4E-04	13.5
XIII.	2.1E-04	1.6E-03	7.5
XIV.	1.0E-04	6.6E-04	6.5
XV.	2.4E-04	1.1E-03	4.5
XVI.	1.1E-04	4.4E-04	3.9
XVII.	1.9E-04	5.8E-04	3.1
XVIII.	7.5E-04	2.2E-03	2.9
XIX.	4.9E-04	1.4E-03	2.9
XX.	2.1E-04	5.4E-04	2.6
XXI.	3.5E-04	7.5E-04	2.1
XXII.	3.6E-04	6.4E-04	1.8
XXIII.	3.8E-04	6.2E-04	1.6
XXIV.	8.2E-05	1.2E-04	1.5

XXV.	2.7E-04	2.2E-04	0.8
* Relative ZIP4 mRNA levels was normalized to that of β-actin and presented as 2^(Ct[β-actin] – Ct[ZIP4]).			

EXAMPLE 3

OVEREXPRESSION OF ZIP4 INCREASES THE PROLIFERATION OF PANCREATIC CANCER CELLS

[0282] To study the potential functions of ZIP4 in pancreatic cancer, three stably overexpressing ZIP4 cell lines were established in MIA PaCa-2 cells (MIA-ZIP4) by using a retrovirus vector (pBabe, Clontech). Parental MIA PaCa-2 cells express less ZIP4 than other pancreatic cancer cell lines (shown in FIG. 1D). Stable cells containing empty vectors (MIA-V) were also established in MIA PaCa-2 cells as controls. Overexpression of ZIP4 in all three MIA-ZIP4 cells was confirmed when compared with MIA-V controls by real-time PCR and Western blotting. ZIP4 overexpression in a representative MIA-ZIP4 stable cell line is shown in FIGS. 2A and 2B (124-fold increase in mRNA). MIA-ZIP4 cells accumulated more zinc than MIA-V cells by 73% ($P < 0.05$, FIG. 2C), indicating that the overexpressed ZIP4 protein was fully functional in transporting zinc ion into the cells. MTS [3-(4,5-dimethylthiazol- 2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay showed that overexpression of ZIP4 in MIA-ZIP4 cells was associated with increased cell proliferation by 101% on day 5, compared with that in MIA-V ($P < 0.01$, FIG. 2D) under a serum-starvation condition but not in the presence of serum. These results indicate that ZIP4 plays an important role in cell proliferation in these pancreatic cancer cells.

[0283] A dose-dependent increase of cell proliferation was found when low concentrations of $ZnCl_2$ were added (in the range of 1–25 μM) to MIA-ZIP4 cells. No significant increase of cell proliferation was found in MIA-V cells upon addition of $ZnCl_2$. However, when a higher concentration of $ZnCl_2$ was added (~50 μM) to MIA ZIP4 cells, cell proliferation was dramatically decreased, probably because of the toxicity of zinc. In contrast, MIA-V cells were less sensitive to zinc than MIA-ZIP4 cells, probably because MIA-V cells take up less zinc than MIA-ZIP4 cells. When exogenous $ZnCl_2$ exceeds 50 μM, both MIA-V and MIA-ZIP4 cells were killed (FIG. 2E).

EXAMPLE 4

ZIP4 PROMOTES PANCREATIC CANCER GROWTH IN THE NUDE MOUSE MODEL OF S.C. XENOGRAFT

[0284] The role of ZIP4 on tumor growth *in vivo* was further analyzed using an immunodeficient nude mouse model. MIA-ZIP4 cells showed a dramatic increase (13-fold) in tumor volume after 6 weeks compared with MIA-V control cells in the s.c. tumor model ($P < 0.01$, FIG. 3A). MIA-ZIP4 cells also significantly increased tumor weight by 30-fold after 6 weeks compared with MIA-V control cells. Tumors from s.c.-injected mice were removed and processed for immunohistochemical analysis. The stability of ZIP4 overexpression in s.c. tumors from the MIA-ZIP4 group was confirmed by real-time RT-PCR. Furthermore, s.c. tumors from MIA-ZIP4-injected mice showed much increased cell proliferation as indicated by the strong positive staining of Ki67, a marker for cell proliferation, compared with that of the MIA-V mice (FIG. 3B). Further analysis of the s.c. tumors for zinc concentration with ICPMS indicated that, overall, 62% more zinc was accumulated in the tumors from the mice implanted with MIA-ZIP4 cells than the tumors from the mice implanted with MIA-V cells ($P < 0.05$, FIG. 3C), indicating that the overexpressed ZIP4 absorbed more zinc, and an increased amount of zinc is necessary for pancreatic cancer progression.

EXAMPLE 5

ZIP4 ENHANCES PANCREATIC CANCER PROGRESSION IN THE NUDE MOUSE MODEL OF ORTHOTOPIC XENOGRAFT

[0285] MIA-ZIP4 cells significantly increased tumor weight by 7.2-fold after 7 weeks compared with MIA-V control cells in the orthotopic model ($P<0.01$, FIGs. 4A–C). Furthermore, mice given injections of MIA-ZIP4 cells showed jaundice (20%), multiple peritoneal dissemination (100%), and severe abdominal ascitic fluid (40%), whereas only 20% of MIA-V control mice showed mild peritoneal dissemination but no other symptoms (FIG. 4C and Table 2). Further analysis of the primary pancreatic tumors for zinc concentration showed that, overall, 80% more zinc was detected in the tumors from the mice implanted with MIA-ZIP4 cells than the tumors from the mice implanted with MIA-V cells ($P < 0.05$, FIG. 4D). Those results

indicate that ZIP4 is a malignant factor that significantly contributes to pancreatic cancer progression *in vivo*.

TABLE 2. ORTHOTOPIC IMPLANTATION OF PANCREATIC CANCER CELLS IN NUDE MICE AT EUTHANIZATION

	MIA-V (N – 5)	MIA-ZIP4 (N = 5)
NO. OF MICE WITH JAUNDICE	0	1
NO. OF MICE WITH PERITONEAL DISSEMINATION	1	5
NO. OF MICE WITH ASCITES	0	2

EXAMPLE 6

SIGNIFICANCE OF EXAMPLES 2-5

[0286] Nutrient uptake and cellular metabolism play essential roles in normal cell cycle and function; therefore, alteration of these events is often associated with cancer. Several studies indicated that zinc transport and metabolism were associated with cancer progression, especially in breast cancer (Kagara *et al.*, 2007; Margaolioth *et al.*, 1983). In the previous examples, it is shown that the overexpression of ZIP4, a zinc importer, was significantly increased in most human pancreatic cancer cell lines and surgical specimens of human adenocarcinoma. Forced overexpression of ZIP4 increased the proliferation of pancreatic cancer cells, and significantly increased tumor growth in both s.c. and orthotopic xenografts of the nude mouse models. Such evidence that zinc transport plays a critical role in pancreatic cancer progression is previously undescribed. Previous studies on zinc concentration in serum and tumor tissues in cancer patients were contradictory. Several reports indicated that levels of zinc in the serum and malignant tissues decreased in liver and prostate cancers (Costello and Franklin, 2006; Chakravarty *et al.*, 1986). However, in breast cancer patients, the zinc levels were decreased in the serum but increased in tumor tissues (Margalioth *et al.*, 1983; Chakravarty *et al.*, 1986; Mulay *et al.*, 1971). This apparent difference between those studies may be due to different tissue and organ sites, which have different mechanisms for zinc uptake (Cousins *et al.*, 2006). Very few studies have been done in connecting zinc metabolism and zinc transporters

with cancer progression. In a recent screening of clinical breast cancer samples for ZIP10 mRNA expression, it was found that ZIP10 was associated with the metastasis of breast cancer to the lymph node, and expression of ZIP10 mRNA was higher in the invasive and metastatic breast cancer cell lines MDA-MB-231 and MDA-MB-435S than in less metastatic breast cancer cell lines MCF7, T47D, ZR75–1, and ZR75–30 (Kagara *et al.*, 2007).

[0287] The data shows that ZIP4 mRNA was significantly increased in 16 of 17 pancreatic cancer specimens and 7 of 8 pancreatic cancer cell lines compared with that in surrounding normal tissues and control HPDE cells, respectively. ZIP4 mRNA expression in pancreatic cancer tissues was also higher than that in chronic pancreatitis tissues. In one embodiment of the invention, ZIP4 is a marker in pancreatic cancer diagnosis. The overexpressed ZIP4 may provide an increased zinc supply to the fast growing tumor cells, in which the zinc availability is limited. Because ZIP4 functions at the plasma membrane (Kim *et al.*, 2004; Eide, 2004; Liuzzi *et al.*, 2004), other proteins or cellular factors may be involved in delivering zinc to its target proteins, which need zinc as an essential component, such as zinc-finger proteins and MMPs. In this regard, it has been shown that murine pancreatic metallothionein, an indicator of zinc homeostasis, is extremely sensitive to zinc availability (Moore *et al.*, 2001).

[0288] Zinc plays an important role in cell growth and proliferation. Zinc deficiency is associated with diverse disorders, such as impaired immune response, growth retardation, delayed wound healing, retarded skeletal development, and osteoporosis (King and Cousins, 2005; Tang *et al.*, 2006). Previous studies have suggested a direct effect of zinc on both proliferation and differentiation of osteoblast-like cells. It has been indicated that zinc regulates cell proliferation through several different mechanisms. It is essential to enzyme systems that influence cell division and proliferation. Depleting zinc from the extracellular milieu results in decreased activity of deoxythymidine kinase and reduced levels of adenosine(5')tetraphosphate(5')- adenosine. In one embodiment of the invention, zinc directly regulates DNA synthesis through these systems. Zinc also influences hormonal regulation of cell division. For example, the pituitary growth hormone (GH)– insulin-like growth factor-I (IGF1) axis is responsive to zinc status.

[0289] Zinc appears to be essential for IGF1 induction during cell proliferation (King and Cousins, 2005; Lee *et al.*, 2003). Based on experiments in which the timing of the zinc

requirement for DNA synthesis in cultured cells was examined, Chesters and Boyne Chesters and Boyne, 1991) hypothesized that zinc was required for the accumulation and maintenance of a protein that mediated the entry of cells into S phase. Those studies suggest that zinc plays an essential role in cell proliferation and growth, possibly through influencing the DNA synthesis and cell cycle. However, a high concentration of zinc is toxic to the cells, and causes apoptosis (Kim *et al.*, 2000). Therefore, cells must have a homeostatic mechanism to maintain the intracellular zinc level within a narrow physiologic range through activity of the zinc transporters.

[0290] The previous examples demonstrate that overexpressed ZIP4 increases pancreatic cancer cell proliferation under serum starvation conditions. A dose-dependent increase of cell proliferation when a low concentration of exogenous $ZnCl_2$ was added (<20 μM) to MIA-ZIP4 cells was also found. No significant increase of cell proliferation was found in MIA-V cells upon addition of $ZnCl_2$ at this range. However, higher concentrations of $ZnCl_2$ (>50 μM) caused dramatically decreased cell proliferation in MIA-ZIP4 cells, whereas MIA-V cells were less sensitive to zinc toxicity than MIA-ZIP4 cells. Those results indicated zinc stimulates cell proliferation in human pancreatic cancer cells at relatively low concentrations. Overexpressed ZIP4 in pancreatic cancer cells MIA PaCa-2 (MIAZIP4) is functional because these cells take up more zinc than MIA-V control cells. In one embodiment of the invention, MIA-ZIP4 cells have better growth potential than MIA-V because of increased intracellular zinc. Relevant to these observations is the up-regulation of ZIP4 expression and plasma membrane localization in intestinal epithelial cells during zinc restriction (Kim *et al.*, 2004; Liuzzi *et al.*, 2004). The negative responsiveness of mouse ZIP4 (mZIP4) to zinc availability in some cell types may prevent the cytotoxicity observed in the MIA-ZIP4 cells upon addition of zinc, where the ZIP4 promoter is not involved.

[0291] Zinc and zinc transporter ZIP10 have also been suggested to be associated with metastatic phenotype of breast cancer cells. Depletion of intracellular zinc and silencing of ZIP10 in invasive breast cancer cells caused a decrease in the migratory activity of these cells, which suggested a positive correlation between zinc import and cancer progression (Kagara *et al.*, 2007). Similarly, ZIP6 (LIV-1) has been associated with estrogen-positive, metastatic breast cancer (Taylor *et al.*, 2003). ZIP4, ZIP6, and ZIP10 are homologs of the nine-member LZT subfamily of ZIP transporters (Cousins *et al.*, 2006). Consequently, the examples showing that

overexpression of ZIP4 significantly enhanced pancreatic cancer progression in both s.c. and orthotopic xenografts of nude mice is of great interest. Overexpression of ZIP4 not only increased the primary tumor size in the orthotopic nude mouse model but also increased the incidence of peritoneal dissemination and ascites in the mice. Further analysis of the s.c. and orthotopic pancreas tumors indicated that more zinc was accumulated in the tumors from the mice implanted with MIA-ZIP4 cells than the tumors from the mice implanted with MIA-V cells. Inside the solid tumor, zinc availability is limited; therefore, overexpression of ZIP4 may provide more zinc for tumor-related proteins that may require zinc, and therefore, support the tumor growth. These examples of the ZIP4 function in human pancreatic cancer demonstrates that overexpression of ZIP4 is associated with enhanced cell proliferation and tumor growth, thereby showing that ZIP4 plays a critical role in human pancreatic cancer progression.

[0292] Different types of parental pancreatic cancer cell lines expressed various levels of endogenous ZIP4. No correlation between the *in vitro* growth and ZIP4 expression levels in these cells was seen. The *in vivo* data from ZIP4 overexpression stable cell lines (MIA-ZIP4) shows that ZIP4 plays a critical role in tumor progression, likely by providing zinc to tumor-related proteins where zinc is limited in the solid tumor.

[0293] In summary, zinc and zinc transporter ZIP4 are markers for pancreatic cancer. Therapies targeting ZIP4 have clinical significance in both human pancreatic cancer and other cancers with high expression of ZIP4.

EXAMPLE 7

MATERIALS AND METHODS FOR EXAMPLES 9-12

[0294] *Chemicals and cell culture.* Human pancreatic cancer cell line ASPC-1 was purchased from the American Type Culture Collection (ATCC, Rockville, MD), and was cultured in RPMI 164 medium with 10% fetal bovine serum (FBS) as previously described (Li *et al.*, 2008; Li *et al.*, 2006). The human ZIP4 (hZIP4) antibody was generated in rabbits against a KLH-conjugated 14-aa synthetic peptide and affinity purified basically as described previously (Liuzzi *et al.*, 2004). Other chemicals were from Sigma (St. Louis, MO).

[0295] *ZIP4 mRNA detection.* The ZIP4 mRNA was analyzed by real time RT-PCR as previously described (Lie t al., 2007; Li *et al.*, 2008). Briefly, real time PCR was performed using the SYBR supermix kit (Bio-Rad, Hercules, CA). PCR reaction included the following components: 100 nM each primer, diluted cDNA templates and iQ SYBR Green supermix, and running for 40 cycles at 95°C for 20 sec and 60°C for 1 min. PCR efficiency was examined by serially diluting the template cDNA and the melting curve data were collected to check PCR specificity. Each cDNA sample was run as triplicates and the corresponding no-reverse transcriptase (RT) mRNA sample was included as a negative control. The β-actin primer was included in every plate to avoid sample variations. The mRNA level of each sample for each gene was normalized to that of the β-actin mRNA. Amount of PCR products was measured by threshold cycle (Ct) values. The relative mRNA level was presented as unit values of $2^{[Ct_{(\beta\text{-actin})} - Ct_{(\text{gene of interest})}]}$. The primer sequences for human ZIP4 gene (SLC39A4) are: Sense 5'-ATGTCAGGAGCGGGTCTTGC-3' (SEQ ID NO: 5); and anti-sense 5'-GCTGCTGTGCTGCTGGAAC-3' (SEQ ID NO:6).

[0296] *Stable cell line selection.* ZIP4 shRNA expressing stable cells were selected in ASPC-1 cells with retrovirus vectors (Origene, Rockville, MD), following manufacturer's instructions. The sequence of the ZIP4 shRNAs used in this study is as follows: 5' ACGTAGCACTCTGCGACATGGTCAGGATG 3' (SEQ ID NO:9). Briefly, ZIP4-shRNA constructs were cotransfected into 293T cells with packing plasmids. Viral supernatants were collected and transduced to the target cells. Stable cell lines expressing ZIP4 shRNA 6 (ASPC-shZIP4) or empty vector (ASPC-shV) were selected with adding 0.5 μg/mL of puromycin into the medium. Three stable cells expressing different shRNAs were selected. These shRNA sequences were #1, 5' TGTCCGTGCGCCAAGCACTGCTGCTGAAC 3' (SEQ ID NO: 7), #2, 5' GGAGAGCGAGGCCTGGATCCTGGCAGTGG 3' (SEQ ID NO: 8), and #3: 5' ACGTAGCACTCTGCGACATGGTCAGGATG 3' (SEQ ID NO: 9).

[0297] *Western blot analysis.* ASPC-shV and ASPC-shZIP4 cells were lysed with ice-cold lysis buffer (20 mM Tris-HCl , pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin and protease inhibitor cocktail) for 30 min in ice. Cell lysates were then collected after centrifugation at 12,000 rpm for 5 min at 4°C. Sixty μg of lysate protein was loaded and total cellular protein was separated with 15% SDS polyacrylamide gel electrophoresis and then

transblotted overnight at 4°C onto Hybond-P PVDF membrane (Amersham Biosciences). The membrane was probed with anti-ZIP4 (1:500) or anti-β-actin (1:3000) antibody at room temperature for 1 h and then washed three times with 0.1% Tween 20-TBS and incubated in a horseradish peroxidase-linked secondary antibody (1:2000) for 1 h at room temperature. The membrane was washed three times with 0.1% Tween 20-TBS and the immunoreactive bands were detected by using enhanced chemiluminescent (ECL) plus reagent kit.

[0298] *Cell proliferation assay.* Cell proliferation was analyzed with the MTS assay. Stable ASPC-1 cells were seeded in 96-well plates (2×10^3 cells/well), and serum-starved (0% FBS) for 24 h. Cell growth was assessed on 1, 2, 3, 4, 5 days after serum starvation. For zinc dependent assay, ASPC-shV and ASPC-shZIP4 cells were treated with a membrane-permeable metal chelator, *N,N,N',N*-tetrakis(2- pyridylmethyl) ethylenediamine (TPEN, Sigma) at 4 μM for 1 h at 37°C. Cells were washed with PBS to remove excess TPEN because TPEN is toxic to cells with long time incubation. Cells were then incubated with DMEM in the presence of 0, 1, and 5 μM $ZnCl_2$. Cell growth was assessed from 1 day after treatment. Twenty μL of MTS reagent mixed with 100 μL growth medium was added to each well, and incubated in 37°C for 2 h. Absorbance was recorded at 490 nm with an EL-800 universal microplate reader (Bio-Tek Instruments, Winooski, VT).

[0299] *In vitro migration and invasion assays.* The cell migration/invasion was determined using a modified Boyden chamber assay. Uncoated poly carbonate inserts (BD Biosciences, San Jose, CA) of 8-μm pore size were either used uncoated or were coated with 100 μL of 100 μg/mL of Matrigel (BD Biosciences). Cells trypsinized and re-suspended in growth media (10^5cells/200μl) were added into the upper compartment of an invasion (matrigel coated insert) or migration (uncoated insert) chamber and 600μl of the same growth media was added into the lower chamber. After 24 hours (for migration) and 48 hours (for invasion) the cells were incubated in Calcein-AM (Molecular Probes, Eugene, OR) for 1 hr at 37°C before fixation. The fluorescence was read from the bottom at an excitation wavelength of 495 nm and emission wavelength of 520 nm. Cells in the upper chamber were then removed, and cells that had migrated or invaded onto the lower surface of the membrane were quantified. The migration/invasion rate was presented as the ratio of the mean fluorescence reading after scraping of the cells divided by the reading before removing the top cells.

[0300] *Pancreatic cancer mouse models.* Subconfluent ASPC-shV and ASPC-shZIP4 cells were harvested by trypsinization, and resuspended in RPMI 1640. Only single cell suspensions with >95% viability were used. The cells (3×10^6) were inoculated either into the right flank (subcutaneous tumor model) or the body of the pancreas (orthotopic tumor model) of 5- to 6-week-old male nude mice (NCI-Charles River). For the subcutaneous tumor model, the tumor size was measured weekly using a digital caliper (VWR international, GA) and the tumor volume was determined with the formula: tumor volume [mm^3] = (length [mm]) × (width [mm])^2 × 0.52. For intrapancreatic injections, mice were anesthetized with 2.5% avertin and a 0.5-1 cm incision was made in the left subcostal region. The 8 tumor cells (3×10^6) in a volume of 50 μL were injected into the body of the pancreas. The peritoneum and skin were closed with a 4.0 surgical suture. After 4 weeks, all surviving mice were euthanized by an overdose of CO_2 exposure and evaluated macroscopically for the presence of orthotopic tumors and the metastases in the abdominal cavity. The orthotopic and metastatic tumor nodules were then explanted, counted and measured. For both subcutaneous and orthotopic experiments, the animals were euthanized when their tumor size reached 2 cm in diameter or the animals became moribund during the observation period, and the time of euthanization was recorded as the time of mortality.

[0301] *Immunohistochemical staining.* Mouse orthotopic tumors were collected and processed into 5 μm slices. Fixed tissue slides were either H&E stained, or incubated with anti-Ki67 antibody (Biodesign International, Cincinnati, OH) for 30 min at 4°C, before DAB visualization, and the sections were then mounted and observed under a phase contrast microscope as described previously (Li *et al.*, 2007).

[0302] *Statistical analysis.* Quantitative results are shown as means ± standard deviations. The statistical analysis was performed by Student's *t* test for paired data between control and treated groups or one way ANOVA for data from multiple groups. A log-rank test was done for comparing the survival curves between the treatment and control groups. $P < 0.05$ was considered statistically significant.

EXAMPLE 8

EXEMPLARY SUMMARY OF EXAMPLES 9-12

[0303] Zinc levels have been correlated with the cancer risk, though the role of zinc and zinc transporters in cancer progression is largely unknown. The zinc transporter, ZIP4, is overexpressed in human pancreatic cancer as shown above. In the following examples, the critical role that ZIP4 plays in a pancreatic cancer xenograft mouse model by silencing ZIP4 expression using shRNA was deciphered. A stably ZIP4-silenced ASPC-1 cell line (ASPC-shZIP4) was established. Silencing of ZIP4 was associated with decreased cell proliferation, migration, and invasion. In the nude mice with pancreatic cancer xenografts, ASPC-shZIP4 showed a significant reduction in tumor volume (62%) and tumor weight (43%) in the subcutaneous model, and decreased orthotopic tumor weight by 34% in the orthotopic model compared with the vector control cells (ASPC-shV). ASPC-shZIP4 also showed reduced incidence of loss of body weight, peritoneal dissemination, jaundice, liver metastasis, lung metastasis, and ascites in the mice. In addition, silencing of ZIP4 downsized the tumor grade from the poorly differentiated to the moderately differentiated compared with ASPC-shV. More importantly, silencing of ZIP4 significantly increased the survival rate of nude mice with orthotopic xenografts ($P = 0.0011$, $n=10$). All ASPC-shZIP4-injected mice (100%) remained alive up to 32 days after tumor implantation, whereas only 30% of the control ASPC-shV-injected mice were alive at the same time point. These results identify a previously uncharacterized role of ZIP4 in pancreatic cancer progression, and demonstrate that knocking down ZIP4 by shRNA is a novel treatment strategy for pancreatic cancers with ZIP4 overexpression.

EXAMPLE 9

SILENCING OF ZIP4 INHIBITS PANCREATIC CANCER CELL PROLIFERATION, MIGRATION, AND INVASION

[0304] Previous examples showed that ZIP4 expression was differentially higher in most human pancreatic cancer cell lines compared with that in the normal human pancreatic duct epithelial (HPDE) cells (Li *et al.*, 2007). ASPC-1 cells were selected to silence ZIP4 by short hairpin RNA (shRNA) because parental ASPC-1 cells express more ZIP4 than other pancreatic cancer cell lines. Three stably over-expressing ZIP4 shRNA cell lines which contained different shRNA sequences were established in ASPC-1 cells (ASPC-shZIP4) using a retrovirus vector (Origene). The sequences used were SEQ ID NO: 7, SEQ ID NO: 8, and SEQ ID NO: 9. Stable

cells containing empty vectors (ASPC-shV) were also established in ASPC-1 cells as controls. Silencing of ZIP4 in the three ASPC-shZIP4 cell lines was confirmed when compared with the ASPC-shV controls by real time PCR and Western blotting. ZIP4 silencing in a representative ASPC-shZIP4 stable cell line was shown in FIG. 5A and 5B (71% reduction in mRNA level, $P < 0.05$).

[0305] To investigate whether silencing of ZIP4 affects the proliferation of ASPC-1 cells, the proliferation of ASPC-shV and ASPC-shZIP4 cells were compared by using a MTS assay. Cells were cultured in zinc sub-optimal conditions, in which the culture medium was pre-incubated with chelex 100 to chelate the zinc ion, and low concentration and high concentration of $ZnCl_2$ were added to the medium, respectively. As shown in FIG. 6A, at low concentration of zinc (1 μM), ASPC-shV cells with wild type ZIP4 showed a growth advantage over ASPC-shZIP4 cells with reduced ZIP4 expression.

[0306] Silencing of ZIP4 in ASPC-shZIP4 cells was associated with 35% decreased cell proliferation by day 3, compared with that in ASPC-shV cells ($P < 0.05$, n=5). In high concentration of zinc (5 μM), no significant growth advantage was observed in ASPC-shV cells compared with ASPC-shZIP4 cells, probably because of the toxicity of high zinc to the cells (FIG. 6B). When the exogenous $ZnCl_2$ exceeds 5 μM, both ASPC-shV and ASPC-shZIP4 cells died quickly after day 2. These results demonstrate that zinc plays an important role in ZIP4 shRNA-mediated growth inhibition. To further study the functions of ZIP4 and to determine the effects of silencing of ZIP4 in vitro, cell migration and invasion assays were performed. As shown in FIG. 6C and 6D, silencing of ZIP4 significantly decreased the cell migrative and invasive abilities of ASPC-1 cells by 68% and 81%, respectively, compared with that in ASPC-shV cells ($P < 0.05$). These results show that ZIP4 plays an important role in pancreatic cancer cell migration and invasion in vitro, which are essential features of pancreatic cancer progression.

EXAMPLE 10

SILENCING OF ZIP4 INHIBITS PANCREATIC CANCER GROWTH IN THE NUDE MOUSE MODEL OF SUBCUTANEOUS XENOGRAFT

[0307] The role of ZIP4 on tumor growth in vivo was further analyzed using an immunodeficient nude mouse model. ASPC-shZIP4 cells showed a delayed onset of tumor growth and dramatic decrease (62%) in tumor volume after 4 weeks compared with the ASPC-shV control cells in the subcutaneous tumor model ($P < 0.001$, n=10; FIG. 7A). ASPC-shZIP4 also significantly reduced subcutaneous tumor weight after 4 weeks compared with ASPC-shV control cells ($P < 0.05$, n=10; FIG. 7B). Mice given injections of ASPC-shV cells had severe skin ulcer (90%), and loss of body weight (50%), whereas only 50% of ASPC-shZIP4 control mice showed mild skin ulcer, but no other symptoms.

EXAMPLE 11

SILENCING OF ZIP4 INHIBITS PANCREATIC CANCER GROWTH AND DOWNSIZES THE TUMOR GRADE IN THE NUDE MOUSE MODEL OF ORTHOTOPIC XENOGRAFT

[0308] Three million cells were injected into the pancreas body of the nude mice. ASPC-shZIP4 cells significantly inhibited orthotopic tumor weight by 34% compared with the ASPC-shV control cells in the orthotopic model (FIG. 8A). Furthermore, mice given injections of ASPC-shV cells showed significant loss of body weight (80%), multiple peritoneal dissemination (100%), jaundice (50%), liver metastasis (40%), lung metastasis (20%), colon/intestine obstruction (40%), and severe abdominal ascetic fluid (40%). On the contrary, only 40% of ASPC-shZIP4 mice showed mild peritoneal dissemination, but no other symptoms (FIG. 8A and Table 3). Tumors from orthotopically injected mice were removed and processed for further histological and immunohistochemical analysis. As shown in Fig 8B and Table 4, the majority of the tumors from ASPC-shV group were poorly differentiated or sarcomatoid, and the tumor area percentage were between 70% and 95% in most tumors, while most of the tumors from the ASPC-shZIP4 group were moderately differentiated, and the tumor area percentage were between 20% to 60%. Furthermore, tumors from ASPC-shZIP4-injected mice showed much decreased cell proliferation as indicated by the staining of Ki67, a marker for cell proliferation, compared with that of the ASPC-shV mice (FIG. 8C). The stability of ZIP4 shRNA in orthotopic tumors from the ASPC-shZIP4 group was confirmed by real time RT PCR, and the expression of ZIP4 was decreased by 63% in ASPC-shZIP4 group compared with that in the

ASPC-shV group at three weeks after tumor implantation. Those results show that ZIP4 is a malignant factor that significantly contributes to pancreatic cancer growth in vivo.

TABLE 3 SYMPTOMS OF NUDE MICE WITH ORTHOTOPIC PANCREATIC TUMORS AT SACRIFICE

	ASPC-SHV (N=10)	ASPC-SHZIP4 (N=10)
# OF MICE WITH LOSS OF BODY WEIGHT	8	0
# OF MICE WITH PERITONEAL DISSEMINATION	10	4
# OF MICE WITH JAUNDICE	5	0
# OF MICE WITH LIVER METASTASIS	4	0
# OF MICE WITH LUNG METASTIASIS	2	0
# OF MICE WITH COLON/INTESTINE OBSTRUCTION	4	0
# OF MICE WITH ASCETIC FLUID	4	0

TABLE 4 SUMMARY OF HISTOLOGICAL STAINING OF ORTHOTOPIC TUMORS

GROUPS	MOUSE ID	TUMOR DIFFERENTIATION (GRADING)*	TUMOR AREA PERCENTAGE (%)
ASPC-SHV	1	M/P	20
	2	P/S	90
	3	M/P	15
	4	P/S	95
	5	P	70
	6	P/S	>95
	7	P/S	95
	8	M	70
	9	P/S	70

ASPC/SHZIP4	10	M/P	50
	11	M	30
	12	M	20
	13	M/P	50
	14	M	60
	15	M	50
	16	M	60

[0309] *M, the moderately differentiated; P, the poorly differentiated; S, the sarcomatoid.

[0310] Three shRNAs (Origene, Rockville, MD) were used to block the expression of ZIP4 in ASPC-1 cells because parental ASPC-1 cells express higher endogenous ZIP4 than most other PC cell lines (FIG. 1D). The expression of ZIP4 in three ZIP4 shRNA transfected ASPC-1 cells was determined by Western blot. Two shRNAs (#1 (SEQ ID NO: 7)and #3 (SE QID NO: 9)) significantly blocked the expression of ZIP4 as compared with the vector control shRNA (FIG. 9A). shRNA #3 (SEQ ID NO: 9) was chosen to construct stable cell lines in ASPC-1 cells (named ASPC-shZIP4) because of the high blocking efficiency. Cells containing empty vector (ASPC-shV) were included as controls. In the s.c. mouse model, it was found that ASPC-shZIP4 cells with reduced ZIP4 expression showed a significant reduction in tumor volume compared with that in the ASPC-shV control group ($p < 0.01$, FIG. 9B). At 4 weeks after tumor implantation, the s.c. tumor volume and tumor weight of the ASPC-shV group were reduced by 62% and 43%, respectively, compared with that in the vector control group (FIG. 9B and 9C). Similarly, in the orthotopic model, ASPC-shZIP4 cells significantly reduced primary tumor weight by 34% after 4 weeks compared with ASPC-shV control cells (FIG. 9D). In the survival study, 10 nude mice were orthotopically implanted with ASPC-shV or ASPC-shZIP4 cells, and survival rate was examined everyday till 32 days. As shown in FIG. 9E, 100% of the ASPC-shZIP4 mice remained alive until 32 days, whereas only 30% of the ASPC-shV mice were alive at that time. The survival rate was significantly increased in the ASPC-shZIP4 group. Those results show that knocking down ZIP4 by shRNA is a novel treatment for PC with high levels of ZIP4.

EXAMPLE 12

SILENCING OF ZIP4 SIGNIFICANTLY INCREASES THE SURVIVAL RATE OF NUDE MOUSE WITH ORTHOTOPIC XENOGRAFTS

[0311] To study the effect of ZIP4 silencing in the survival of the mice, ten nude mice were orthotopically implanted with ASPC-shV or ASPC-shZIP4 cells, and the survival rate was examined everyday up to 32 days. As shown in FIG. 8D, 100% of the ASPC-shZIP4 mice remained alive up to 32 days, whereas only 30% of the ASPC-shV mice were alive at the same time point. The survival rate was significantly increased in the ASPC-shZIP4 group ($P = 0.0011$, $n=10$). Those results demonstrate that knocking down ZIP4 by shRNA is an effective method to control pancreatic cancer growth, and represents a novel and potent treatment for ZIP4-overexpressed pancreatic cancer.

EXAMPLE 13

SIGNIFICANCE OF EXAMPLES 9-12

[0312] Recent studies and the Examples 2-6 showed that zinc transport and zinc homeostasis play important roles in cancer progression, especially in pancreatic cancer and breast cancer (Kagara *et al.*, 2007; Li *et al.*, 2007; Margalioth *et al.*, 1983). However, the molecular mechanism by which zinc transporters regulate cancer growth remains unknown, and it has not been previously described whether silencing of zinc transporters has any impact on tumor growth.

[0313] Cells must have a fine homeostatic mechanism to maintain the intracellular zinc level within a narrow physiologic range through activity of the zinc transporters. In pancreatic cancer cells, zinc stimulates cell proliferation at low concentrations. Previous examples found a concentration-dependent increase of cell proliferation when less than 20 μM of exogenous $ZnCl_2$ was added to MIA-ZIP4 cells, a pancreatic cancer cell (MIA PaCa-2) that stably overexpressed ZIP4. Higher concentrations of $ZnCl_2$ (more than 50 μM) caused a dramatic decrease in cell proliferation (Li *et al.*, 2007). In ASPC-1 cells, the zinc concentration which stimulates cell proliferation is even lower. In zinc sub-optimal conditions, in which the medium was pretreated with chelex 100 to remove the zinc ion, and low concentration of $ZnCl_2$ (1 μM)

was added, ASPC-shV cells with wild type ZIP4 had a significant growth advantage over ASPC-shZIP4 cells with reduced ZIP4 expression. When incubated with a high concentration of zinc (5 μM), no significant growth advantage was observed in ASPC-shV cells compared with ASPC-shZIP4 cells, probably because of the toxicity of high zinc to ASPC-1 cells. When the exogenous $ZnCl_2$ exceeds 5 μM, both ASPC-shV and ASPC-shZIP4 cells died quickly after 2 days. These results show that zinc and zinc transporter ZIP4 play important roles in pancreatic cancer cell proliferation. Silencing of ZIP4 was also associated with decreased cell migration and invasion, which strongly suggests that ZIP4 not only regulates pancreatic cancer cell proliferation, but also has an impact on cancer metastasis. Previous studies have shown that another zinc transporter, ZIP10, is a malignant factor and is associated with metastatic phenotype of breast cancer cells. Depletion of intracellular zinc and silencing of ZIP10 in invasive breast cancer cells caused a decrease in the 13 migratory activity of these cells, suggesting a positive correlation between zinc transport and breast cancer metastasis (Kagara *et al.*, 2007). Similarly, ZIP6 (LIV-1) has been indicated to play an important role in estrogen-positive, metastatic breast cancer (Taylor *et al.*, 2003). ZIP4, ZIP6 and ZIP10 are homologs of the nine member LZT subfamily of ZIP transporters (Cousins *et al.*, 2006), these studies suggest a novel function of zinc transporters of the LZT subfamily in cancer metastasis and progression.

[0314] Consequently, the above examples showed that silencing of ZIP4 caused an inhibition of pancreatic cancer growth in both subcutaneous and orthotopic xenografts of nude mice. ASPC-shZIP4 cells showed a delayed onset of tumor growth and dramatic decrease in tumor volume and tumor weight in the subcutaneous model. In the orthotopic model, silencing of ZIP4 was associated with significant inhibition of orthotopic primary tumor weight, and reduction of the incidence of loss of body weight, peritoneal dissemination, jaundice, liver metastasis, lung metastasis, colon/intestine obstruction, and abdominal ascetic fluid. Further histological analysis indicated that silencing of ZIP4 led to reduced tumor grades, and thereby in one embodiment of the invention silencing of ZIP4 sensitize the tumors to additional therapies. Another exciting finding of these examples is that silencing of ZIP4 increased the survival rate in nude mice with orthotopic xenografts. Those results indicate that knocking down ZIP4 by shRNA is an effective method to control pancreatic cancer growth, and represents a novel and potent treatment for ZIP4-overexpressed pancreatic cancer. By defining the ZIP4 profile in pancreatic cancer before and after therapy, the ZIP4 profile can be used to guide the choice of initial and sequential ZIP4-based therapies. Individuals with different expression of ZIP4 may

respond to the ZIP4 shRNA therapy differently. Considering the short life span of shRNA, it might be necessary to modify the shRNA or use multiple cycles of shRNA treatment (Liu *et al.*, 2008). This concept is important, since multiple treatment cycles may be used to have an even further impact on survival in patients with metastatic pancreatic cancer; and sequential therapy could be guided by the ZIP4 profile. To increase the efficacy of shRNA therapy, combinational therapy (double or triple therapy) may represent a novel strategy for pancreatic cancer treatment. The studies show that silencing of ZIP4 downsized tumor grade, and may sensitize the tumors to subsequent treatment. Therefore, the combinational therapies with ZIP4 shRNA and chemo/radiation therapy may increase the efficacy of individual therapy. In summary, zinc transporter ZIP4 is a marker for pancreatic cancer. New therapies targeting ZIP4 have high clinical significance in both human pancreatic cancer and other cancers with high expression of ZIP4.

EXAMPLE 14

EXPRESSION OF ZIP4 IS HIGHER IN PANCREATIC CANCER TISSUES THAN IN SURROUNDING BENIGN TISSUES

[0315] Expression of ZIP4 protein in more than 20 tumor samples along with their surrounding benign tissues was also examined. The tumor samples showed specific positive staining of ZIP4 on the membrane of cancer cells in the pancreatic adenocarcinoma, while the surrounding benign or pancreatic intraepithelial neoplasia (PanIN) lesions next to the duct adenocarcinoma showed negative staining of ZIP4 as in FIG. 10. Thus, high expression of ZIP4 in the majority of PC tissues and cell lines shows this zinc transporter contributes to PC growth.

EXAMPLE 15

OVER-EXPRESSION OF ZIP4 IN MIA PACA-2 CELLS INCREASES PHOSPHORYLATION OF STAT3

[0316] In order to understand the molecular mechanisms of ZIP4-induced cell proliferation, and tumor growth, the expression and phosphorylation status of several transcriptional factors was determined. As shown in FIG. 11, phosphorylation of STAT3 but not

others was significantly increased in MIA-ZIP4 cells compared with that in MIA-V cells. In one embodiment of the invention, ZIP4-induced phosphorylation of STAT3 may be responsible for the stimulation of cell proliferation in PC cells with high ZIP4 levels.

EXAMPLE 16

ZIP4 UPREGULATES THE EXPRESSION OF CYCLIN D1 IN PANCREATIC CANCER XENOGRAFTS

[0317] To delineate the related signaling pathway of ZIP4 and STAT3, a microarray analysis in xenografts (orthotopic model) from MIA-V and MIA-ZIP4 cell-injected nude mice was done by using the comprehensive human Genome U133 PLUS 2.0 array chips (Affymetrix, Santa Clara, CA). It was found that 510 genes were upregulated more than 2 folds, and 591 genes were downregulated more than 2 folds. Both regulation and down regulation were statistically significant. Among these upregulated genes, it was found that cyclin D1 expression was increased by 2.6 folds in MIA-ZIP4 group compared with that in MIA-V group (FIG. 12A). The microarray data was confirmed with quantitative real time RT PCR in the tissues from the orthotopic xenografts, and a 2.5 fold-increase of cyclin D1 mRNA level in the MIA-ZIP4 group compared with that in the MIA-V group was found (FIG. 12B), which is consistent with the microarray data. In ASPC-1 cells, silencing of ZIP4 by shRNA caused a 64% reduction of cyclin D1 mRNA compared with that in vector control cells. Cyclin D1 is a downstream target gene of STAT3, and is a key molecule controlling cell proliferation and survival in cancers. In one embodiment of the invention, the downstream pathway of ZIP4-mediated cell proliferation and tumor progression is through activation of STAT3 and upregulation of cyclin D1 in PC.

EXAMPLE 17

ZIP4 UPREGULATES THE EXPRESSION OF IL-6 IN PANCREATIC CANCER XENOGRAFTS

[0318] IL-6 is a known upstream activator for STAT3, in order to elucidate the signaling pathway of ZIP4 and STAT3, the expression of IL-6 in xenografts from MIA-V and MIA-ZIP4 cell-injected nude mice was examined by real time PCR. IL-6 level was significantly

increased in the MIA-ZIP4 group of the orthotopic xenografts by 23.7 folds compared with that in the MIA-V group (FIG. 13A). IL-6 was also increased in the MIA-ZIP4 group of the s.c. xenografts by 8.8 folds compared with that in the MIA-V group (FIG. 13B). IL-6 is one of major proinflammatory cytokines, and an activator of STAT3 phosphorylation. In one embodiment of the invention, IL-6 is an important intermediate signaling molecule connecting ZIP4 and STAT3 pathways in PC.

EXAMPLE 18

ZINC-DEPENDENT PANCREATIC CANCER CELL PROLIFERATION

[0319] To determine if ZIP4 mediated PC cell growth is zinc-dependent, MIA-V and MIA-ZIP4 cells were treated with different doses of zinc and the cell proliferation was examined. A dose-dependent increase of cell proliferation was found when a low concentration of $ZnCl_2$ was added (in the range of 1-25 μM) to MIA-ZIP4 cells. No significant increase of cell proliferation was found in MIA-V cells upon addition of $ZnCl_2$. However, when a higher concentration of $ZnCl_2$ was added (~50 μM) to MIA-ZIP4 cells, cell proliferation was dramatically decreased, probably because of the toxicity of zinc. In contrast, MIA-V cells were less sensitive to zinc than MIA-ZIP4 cells, possibly because MIA-V cells take up less zinc than MIA-ZIP4 cells. When exogenous $ZnCl_2$ exceeds 50 μM, both MIA-V and MIA-ZIP4 cells were killed (FIG. 14). In one embodiment of the invention, zinc plays an important role in PC growth, adequate zinc promotes PC cell proliferation, and low zinc may slow down PC cell proliferation. This provides a dietary recommendation for PC patients emphasizing a low zinc diet such as more vegetable and fruit than meat. In one embodiment of the invention, a low zinc diet is used in combination with the ZIP4 inhibitory agent.

EXAMPLE 19

LIPOSOME/DNA DELIVERY IN VIVO

[0320] The success of a shRNA therapy is highly relying on the transfection efficiency and the stability of the shRNA. In studies, liposomes are used to deliver shRNAs or other genes into the animal for shRNA therapy or gene. It was found that 3 cycles treatment of liposomal/human PDX-1 shRNA reduced the expression of PDX-1 in PC cells, and significantly

inhibited PC growth in immunodeficiency mice with 4/5 mice tumor-free, compared to the group control, in which all mice had large volumes of tumor in an immunodeficient mouse model (Li *et al.*, 2008; FIG. 15). These exciting data suggest PC growth can be inhibited with direct targeting of specifically over-expressed genes in PC by 3 cycles treatment of iv liposome/ shRNA, and demonstrate the feasibility of a novel shRNA therapy targeting ZIP4 in PC.

EXAMPLE 20

TRANSGENIC KRAS MOUSE MODEL OF PANCREATIC CANCER

[0321] To further evaluate the shRNA therapy or combinational therapy targeting ZIP4 in PC, it is necessary to test the efficacy of these treatments in a more robust animal model, such as a transgenic mouse model. Most available animal models for PC are Kras transgenic mice, in which Kras gene was activated. These mice usually develop pancreatic intraepithelial neoplasia (PanIN) lesions, which is thought to be a precursor of PC (Hingorani *et al.*, 2003). In combination of Kras activation with loss of tumor suppressor gene such as p53 in mice develops PC that resembles the genetic and pathologic profile of human PC (Bardeesy *et al.*, 2006; Hingorani *et al.*, 2005). A Kras transgenic mouse model was recently developed (MDA mouse model), in which high levels mutant Kras was expressed specifically in pancreatic acinar cells under a CMV promoter. The high expression of Kras led to the rapid development of fibrosis and loss of acinar cells creating a condition resembling chronic pancreatitis and to abundant multifocal mPanINs that rapidly progressed to invasive and metastatic PC. As shown in FIG. 16A, in younger mice, the mPanINs were primarily mPanIN-1 and occasionally mPanIN-2 lesions. As the animals aged, there was an increase in the level of dysplasia and in the frequency of mPanIN 3 lesions. At 4 months of age, one mouse had developed PC (FIG. 16B) with metastasis to the liver (FIG. 16C) and. By 4 to 6 months of age, 29% (7/24) of mice had developed PC. At 9 months, PC incidence increased to 50% (10/20). This transgenic mouse model is an excellent tool to study the pathogenesis and progression of PC.

[0322] Mouse pancreas tissues was collected at different stages from the Kras transgenic mice, and the expression of mouse ZIP4 (mZIP4) protein was examined by immunohistochemistry. As shown in FIG. 17, mZIP4 staining was strong positive in mouse PC tissues, but was only weak positive or negative in normal pancreatic duct. These results show that mZIP4 is a good molecular target for PC treatment.

EXAMPLE 21

SIGNIFICANCE OF PREVIOUS EXAMPLES

[0323] ZIP4 expression was significantly increased in most human PC tissue specimens and cell lines. Forced over-expression of ZIP4 increased the cell proliferation and tumor growth. Silencing of ZIP4 inhibits pancreatic tumor growth and significantly increases survival rate in the nude mice xenograft model. STAT3 was activated in ZIP4 over-expressing MIA PaCa-2 cells, and IL-6 and cyclin D1 expression were also upregulated by ZIP4 over-expression in PC xenografts. In one embodiment o the invention, aberrant expression of ZIP4 plays a critical role in PC growth, and the genomic profile of ZIP4 can guide the choice of therapy in PC. Feasibility of this invention is demonstrated by the previously shown data and in recent publications (Feurino *et al.*, 2007; Li *et al.*, 2007; Liu *et al.*, 2008; Li *et al.*, 2004; Li *et al.*, 2006; Li *et al.*, 2007; Fu *et al.*, 2006; Li *et al.*, 2008).

EXAMPLE 22

SUMMARY AND MATERIALS AND METHODS FOR EXAMPLES 23-24

[0324] In the previous examples, ZIP4 expression in 17 pairs of PC specimens by real time RT PCR and immunohistochemistry was examined, and it was found that ZIP4 mRNA levels in 94% of the PC tissues were substantially up-regulated as compared with that in the surrounding normal tissues. ZIP4 protein levels were also specifically increased in the PC tissues as indicated by immunohistochemistry. To understand more details of ZIP4 involvement in PC, a larger number of human PC tissue samples (200) are used to study the aberrant ZIP4 expression profiles and the correlation with different stages of human PC are also studied. In one embodiment of the invention, an individuals ZIP4 expression profile is used to determine the appropriate cancer therapy for the individual, such as the dosage of ZIP4 inhibitor to administer, or the combination of anti-cancer therapy to administer.

[0325] Many genes that are aberrantly expressed in cancers including PC often have mutations or SNPs. These mutant forms may play important roles in cancer pathogenesis and progression. Therefore, the genetic variations (mutations and SNPs) of ZIP4 gene are determined by Sanger sequencing in the PC specimens. Besides the previous finding of the

function of ZIP4 in PC cell proliferation and tumor growth in MIA PaCa-2 and ASPC-1 cell lines, the function of ZIP4 in PC growth in other PC cells in addition to MIA PaCa-2 and ASPC-1 is unknown.

[0326] ZIP4 expression levels is compared to different stages of human PC. 200 pancreatic adenocarcinoma tissues is examined. In addition, 10 normal pancreases from organ donors and autopsy cases, and 20 chronic pancreatitis specimens are examined. ZIP4 mRNA expression levels are determined by using real-time RT-PCR, ZIP4 protein expression levels by using western blot and immunohistochemistry followed by quantum dot (QDot) deconvolution imaging analysis. The criteria to correlate the ZIP4 expression with the different stages of PC progression is also calculated. In one embodiment of the invention, the expression levels of ZIP4 is used to indicate what stage of PC the individual has.

[0327] *Sample collection:* Patients from any age, gender, religious affiliation, and race are recruited for the study. The Elkins Pancreas Center tissue bank currently contains more than 200 PC specimens, and a complete matching set of surrounding normal pancreas, plasma, serum, and blood cells from the same patient on an approved human protocol at BCM. The predictive potential of ZIP4 are tested as a new molecular target in human PC tissues. The tissue samples for this study are immediately available in both formalin fixed blocks and frozen storages.

[0328] All pancreatic tissues in paraffin sections are processed and evaluated with H&E staining. The tissue quality and structure are initially studied. For PC, histopathological grade and clinical staging are evaluated according to the criteria by National Cancer Institute (NCI) and the International Union Against Cancer (UICC) TNM classification (Argani *et al.*, 2001; Rubben *et al.*, 1979). These tissues are analyzed with pathology standards. Human pancreatic adenocarcinoma and surrounding normal tissues are collected and processed. Detailed studies of carefully microdissected clinical samples of normal pancreas, pancreatic intraepithelial neoplasia (PanIN 1-3), primary and metastatic human PC using quantitative RT-PCR, immunohistochemistry, and western blot analysis are performed.

[0329] *Detection of ZIP4 mRNA in microdissected specimens using quantitative RT-PCR.* Samples of human PC are obtained from patients undergo surgery. Neoplastic tissues as well as normal pancreas from the margin of resection are maintained in a tissue bank which is

associated with a prospective clinical database recording demographic, staging, treatment, and outcome data. Tissues of both primary and metastatic cancer have been obtained whenever possible. Samples of "normal" pancreas surrounding the tumor often contain preneoplastic lesions called pancreatic intraepithelial neoplasia. Normal pancreas from organ donors is also available. Samples are assayed for expression of ZIP4 mRNA using RT-PCR. Specimens are immediately frozen in liquid nitrogen in the operating room and sectioned using a microtome and placed on glass slides. Cell clusters representing pancreatic adenocarcinoma or pancreatic intraepithelial neoplasia are identified using established histologic criteria and dissected using a PixCell laser capture microdissection (LCM) with an infrared diode laser (Arcturus Engineering, Santa Clara, CA). 5 μm thick serial sections of retrieved fresh frozen tissues are cut and immediately stained using a HistoGene™ LCM Frozen Section Staining Kit (Arcturus) and LCM is performed immediately thereafter. The RNAs of target cells from 10 serial frozen sections are isolated from a laser beam. LCM used in this project is critical because PC specimens are highly heterogeneous. The admixture of contaminating bystander cells might interfere with the results of quantitative PCR analysis. Therefore, pure tumor cell populations have to be isolated in order to obtain reliable data. Total RNA is isolated from the specimens using an Ambion "RNAqueous-4PCR" kit following the manufacture's instructions as previously described (Li *et al.*, 2004; Li *et al.*, 2006). Specific primers for ZIP4 gene are used as follows: Forward primer, 5'ATGTCAGGAGCGGGTCTTGC3' (SEQ ID NO: 5), reverse primer, 5'GCTGCTGTGCTGCTGGAAC 3' (SEQ ID NO: 6). The mRNA levels for ZIP4 are analyzed by real-time RT-PCR using a Bio-Rad iCycler system (Bio-Rad, Hercules, CA) as previously described (Li *et al.*, 2004; Li *et al.*, 2006). The real-time PCR is performed by using a SYBR supermix kit, and running for 40 cycles at 95°C for 20 secs and 60°C for 1 min. The β-actin primers (Forward, 5' CTGGAACGGTGAAG GTGACA 3' (SEQ ID NO: 10), and reverse, 5' AAGGGACTTCCTGTAACAATGCA 3' (SEQ ID NO: 11) are included in every plate to avoid sample variations. The mRNA level of each sample for each gene are normalized to that of the β-actin mRNA. Relative mRNA level are presented as 2^[Ct(β-actin) - Ct(gene of interest)]. All experiments may be repeated at least three times.

[0330] *Detection of ZIP4 protein expression using immunohistochemistry and western blot.* The presence of ZIP4 proteins is determined using immunohistochemical analysis. The human ZIP4 antibody (Ab) generated in rabbits against a KLH-conjugated 14-aa synthetic peptide and affinity purified as described previously (Li *et al.*, 2007; Liuzzi *et al.*, 2004) is used

to detect the expression of ZIP4 protein. To validate the specificity of ZIP4 immunoreactivity, the following controls are included: 1) preimmune serum in place of the primary antibody; and 2) primary Ab absorbed with excess blocking peptide. Clinical samples of normal pancreas, pancreatic intraepithelial neoplasia, primary invasive pancreatic adenocarcinoma, and metastatic tumors are fixed in 4% formaldehyde and embedded in paraffin. Sections (5 μm) are deparaffinized and incubated in 1% BSA and 5% normal goat serum in TBS for 1 hour at room temperature and then be incubated with ZIP4 antibody (affinity-purified and diluted 1:2000 in TBS plus 1% dry milk) for overnight at 4°C followed by incubation with appropriate secondary antibody conjugated with different types of QDots, such as 655 QDots (blue), 605 QDots (red) or 565 QDots (green). The QDot nanocrystals have unique optical properties that create an extremely bright, photo-stable signal and can be used for multiplexing. Another advantage of using QDot is that it can detect weak signals in tissue samples because of its capacity to recognize and bind to antigens with low expression. Dual or triple labeling with QDots are used if necessary. Imaging is performed using the multispectral deconvolution camera and software from Nuance/Optimas system (CRI, Inc., Woburn, MA). The images are evaluated using image analysis software in a blinded manner relative to the clinical follow-up data including recurrence and death to establish whether ZIP4 has additional predictive value for PC. The following criteria of ZIP4 expression is used based on previous experience with QDot staining: high expression>250 intensity units (iu); intermediate expression 100-250 iu; and low ZIP4 expression <100 iu. ZIP4 expression in PC is correlated with NCI grades and UICC TNM classification and is compared to its surrounding normal pancreatic ductal structures. ZIP4 expression is also compared among PC, normal pancreas structure, and chronic pancreatitis tissues as described above.

[0331] To confirm the expression of ZIP4 in human PC tissues, western blot analysis is also performed. Proteins are extracted from above formalin-fixed paraffin-embedded (FFPE) tissue sections using a QIAGEN Qproteome FFPE Tissue Kit (Valencia, CA) as suggested by manufacture's instructions (Shi *et al.*, 2006). Total protein is separated with 10% SDS-polyacrylamide gel electrophoresis and then transblotted overnight at 4°C onto Hybond-P PVDF membrane (Amersham biosciences, Piscataway, NJ). The membrane is probed with anti-ZIP4 (1:200) or anti-β-actin (1:3000) antibody at room temperature for 1 h and then washed three times with 0.1% Tween 20-TBS and incubated in a horseradish peroxidase-linked secondary antibody (1:2000) for 1 h at room temperature. The membrane is washed three times

with 0.1% Tween 20-TBS and the immunoactive bands are detected by using enhanced chemiluminescent (ECL) plus reagent kit.

The correlation of ZIP4 expression with patient outcome, performance, and overall survival is calculated in a cohort of 200 PC patients and 20 pancreatitis patients treated over the past four years. Nonparametric Spearman's rank-order correlation coefficients are generated to determine if any correlations exist between ZIP4 staining grade and staging of PC and pancreatitis. Survival is measured in a univariate fashion for each variable using the Kaplan-Meier procedure. A stratified Kaplan-Meier procedure is also performed by first analyzing by clinical stage, histology, age or location of metastatic spread, then analyzing by degree of ZIP4 expression. SPSS software (version 11.0, Chicago, IL) is used for statistical analyses. In one embodiment of the invention, an idivudals overall survival rate is determined by comparing the individuals ZIP4 mRNA expression levels to a standard range of ZIP4 expression levels.

EXAMPLE 23

DETERMINATION OF GENETIC VARIATION OF ZIP4 IN PANCREATIC CANCER TISSUES USING SANGER SEQUENCING

[0332] *DNA sequencing*. To further determine if there are mutations or SNPs in ZIP4 gene *SLC39A4*, the PC tissue samples and the surrounding normal tissues are sequenced. The same patient cohorts in Example 22 are used for the DNA. For genomic DNA, tissue samples are processed and the DNA is extracted using the QIAamp DNA Mini kit (Qiagen). The blood samples from the patients are collected in PAXgene Blood DNA tubes and the DNA is isolated with the PAXgene Blood DNA kit (PreAnalytiX). The 12 exons and their flanking sequences of ZIP4 gene have already been identified and their corresponding primer sets have been designed to generate amplicons between 200-420 bases. Every effort has been made during the designing of the primers to ensure full-length sequencing of the amplicon in both directions. Large exons are covered by several overlapping amplicons. To allow the sequencing of the different amplicons with a single reverse or forward primer, universal terminal reverse (CTGCTCAGGAAACAGCTATGAC, SEQ ID NO: 12) and forward (CTCGTGTAAAACGACGGC CAGT, SEQ ID NO: 13) linkers were incorporated in the design of the primers. Primer efficiency is tested on commercial human genomic by PCR and

sequencing. PCR reaction consists of 40 cycles of a denaturation step at 94°C for 15 secs, annealing at 64°C for 30 secs, and an extension step at 72°C for 45 secs. The above conditions are modified for primer sets with lower efficiency to obtain sufficient PCR products for sequencing. A 2 μl PCR reaction mixture is loaded on an agarose gel to check the quality of the PCR products. The purified PCR product is diluted and sequenced using BigDye Terminator Cycle Sequencing kit and loaded on ABI 3700 DNA Sequencer. Most of the sequencing preparation is performed by robot. The PCR primer pairs, PCR reaction mix, and DNA is arrayed in 96-well format plates allowing the PCR reaction set up by automated pipettors into 384-well plates. A LIMS (Laboratory Information Mgmt Systems) and bar-code systems allows the tracking of the sample throughout the data pipeline.

[0333] *Sequence analysis*. Sequencing data is assembled with the GS Amplicons Variant Analysis software version 1.0.53 that aligns the reads against a defined target reference sequence. With this software, the sequencing data are expressed as a signal intensity proportional to the number of bases added and plotted as a function of flow order. The reference sequence is also converted into an idealized flowgram. Variants are identified from the alignment, quantitated, and presented in tabular form and variation plot. The automated detection of the mutations is performed by analytical software developed at BCM HGSC. Sequence data is compared with the dbSNP database to determine if the identified mutations are novel, and then validated with the TaqMan Genotyping Assay. Custom TaqMan SNP Genotyping Assay consists of two sequence specific primers for amplifying the polymorphic sequence of interest and two probes with different fluorescent dyes for distinguishing between the two alleles. The putative somatic mutations are identified within and surrounding each exon by comparing the variant lists obtained in the tumor DNA pool with the patient normal pool. Higher emphasis is first put on somatic nonsynonymous mutations with an allelic frequency above 0.25%. Somatic non-coding mutations is carefully considered: mutations in introns, at the splice junction of exons, is also selected. Base variations between the reference sequence and the normal/tumor sequence are not eliminated automatically as polymorphisms since they might be hereditary and predispose to pancreatic adenocarcinoma. The dbSNP database is searched for these variations to determine if they are novel or already described as polymorphisms.

EXAMPLE 24

DETERMINE ZIP4 EXPRESSION LEVELS EFFECT ON TUMOR PROGRESSION IN DIFFERENT PANCREATIC CANCER CELL LINES

[0334] The previous example data showed that forced over-expression of ZIP4 in MIA PaCa-2 cells significantly increased PC cell proliferation and tumor growth, and silencing of ZIP4 in ASPC-1 cells inhibited PC progression. The specific role of ZIP4 in PC pathogenesis may also be determined. Comprehensive experiments are designed to examine the effect of ZIP4 over-expression and silencing in cell proliferation and tumor growth in several other PC cell lines including BxPC-3, Capan-1, and Capan-2 cells. Furthermore, DNA from those PC cell lines and HPDE cells are isolated. As described above, Sanger sequencing is performed on those cells to test for ZIP4 genetic variations. In order to address the correlation between any genetic variation of ZIP4 and PC cell behavior, mutant ZIP4 constructs are generated based on the findings in Example 23 to confirm whether mutant forms of ZIP4 specifically affects PC cell behavior. In one embodiment of the invention, the type of cancer cell determines the type of therapy given, for example, the dosage of ZIP4 inhibitor administered to the individual, or the additional types of anti-cancer therapy given.

[0335] BxPC-3, Capan-1, and Capan-2 stable cell lines are constructed, in which ZIP4 is over-expressed or silenced by shRNA. Point mutations of ZIP4 (as identified in Example 23) are included by site-directed mutagenesis as described previously (Li *et al.*, 2001). BxPC-3, Capan-1, and Capan-2 cells are well characterized duct epithelial cells. These three cell lines are chosen to study the function of ZIP4 because they represent the diversity of PC cells, and are derived from different origins and have different genetic background. BxPC-3 cells are derived from primary tumor, and have wild type Kras. Capan-1 cells are from liver metastasis. Capan-2 cells have wild type p53. The parental Capan-1 cells express relatively low levels of ZIP4, BxPC-3 cells express intermediate levels of ZIP4, while Capan-2 express high levels of ZIP4, therefore, ZIP4 is over-expressed in Capan-1 and BxPC-3, and knock down ZIP4 in Capan-2 and BxPC-3 cells, by retrovirus vectors, are made as described previously (Li *et al.*, 2007; Li *et al.*, 2008), to determine if alteration of ZIP4 expression and/or genetic variation of ZIP4 in human PC cells affects the cell proliferation *in vitro* and tumor growth in the immune deficient mice. The empty vector controls and non-related GFP gene are included as negative controls in all three cell lines.

[0336] *Cell proliferation assay.* Stable Capan-1, BxPC-3, and Capan-2 cells over-expressing wild type or mutant ZIP4 gene or ZIP4 shRNA are used to study the cell proliferation, and cells expressing vector or GFP controls are included as well. The cell proliferation is measured by MTS assay (an improved MTT assay, due to the solubility of the MTS formazan product in tissue culture medium), which measures the dehydrogenase enzyme activity found in metabolically active cells. Since the amount of dehydrogenase is proportional to the number of living cells, the intensity of the produced color (readout for the MTS assay) is a good indication of the viability of the cells, and correlates well with the cell numbers. Briefly, the cells mentioned above are seeded in 96-well plates (2×10^3 cells/well), and serum starved for 24 hrs. Cell growth are assessed at 0, 1, 2, 3, 4, 5, and 6 days after serum starvation. Culture medium are removed and 20 μl of MTS reagent (Promega, WI) mixed with 100 μl of complete growth medium are added to each well of a 96-well plate, and incubated for 2 hrs at 37°C in dark. The absorbance at 490 nm is detected using an EL-800 universal microplate reader (SCIMetrics, TX). All samples are triplicated, and all experiments are repeated at least three times. In addition to MTS assay, [^{3}H]thymidine incorporation assay is also used as an alternative method to examine the cell proliferation because of its unique advantage in determining the index of DNA replication in a fast and sensitive way, and has been widely used for detecting cell proliferation. The stable cell lines mentioned above are seeded in 96-well plates (2×10^3 cells/well) and starved in serum free medium for 24 hrs. One μCi/ml [^{3}H]thymidine is added to each well 4 hrs before collecting the cells. [^{3}H]thymidine incorporation is measured in scintillation solution using a microplate scintillation & luminescence counter as described in previous publications (Li *et al.*, 2007; Li et al, 2004; Li *et al.*, 2008).

[0337] *In vivo tumor model.* To examine the effect of ZIP4 on pancreatic tumor growth, immune deficient mice are injected stable Capan-1, BxPC-3, and Capan-2 cells over-expressing wild type or mutant ZIP4 gene or ZIP4 shRNA, and cells expressing vector controls are included as well. The tumor formation is evaluated and it is determined whether alteration of ZIP4 expression or genetic variation of ZIP4 in human PC cells affect tumor growth in the immune deficient mice. Six to eight weeks old male athymic nude mice are used for subcutaneous (s.c.) and orthotopic implantation. The mice are purchased from the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD) and are maintained under specific pathogen-free conditions. PC cells at 80% confluence are trypsinized to produce single cell suspensions. Cells are washed with PBS, spun in a centrifuge, and resuspended in PBS at a

concentration of 2×10^7 viable cells per ml (as determined by trypan blue exclusion). For s.c. injection, the tumor cells ($1\text{-}3\times10^6$ in 0.1 mL of PBS) are then injected into the right flank of the nude mice. A total of 30 mice per group are used. Seven days after the injection and every third day thereafter, the length and width of the tumors that developed are measured. Mice are sacrificed at 60 days by an overdose of CO_2 exposure (Li *et al.*, 2007; Arumugam *et al.*, 2005), and final tumor mass and volume are also recorded. Tumor volume aree calculated as (length / 2) $\times$ (width^2), and tumor mass are weighted. For orthotopic injection, mice are anesthetized with 2.5% avertin and a 0.5-1 cm incision are made in the left subcostal region. The tumor cells ($1\text{-}3\times10^6$) in a volume of 50 μL are injected into the body of the pancreas. The peritoneum and skin are closed with a 4.0 surgical suture. After 4 weeks, all surviving mice are euthanized by an overdose of CO_2 exposure and evaluated macroscopically for the presence of orthotopic tumors and the metastasis in the abdominal cavity. The orthotopic and metastatic tumor nodules are then explanted, counted and measured. For both s.c. and orthotopic experiments, the animals are euthanized when their tumor size reach 2 cm in diameter or the animals become moribund during the observation period, and the time of euthanization is recorded as the time of mortality.

[0338] All tumor tissues are collected and immunohistochemistry analysis is performed to further determine the proliferation, and vascularity of those PC cells in mice (i.e. Ki67, and CD31 staining). Rabbit anti-human Ki67 and anti-mouse CD31 Abs are obtained from Biodesign International (Cincinnati, OH), and the staining is done as described previously (Li *et al.*, 2007).

[0339] *Microarray analysis of PC orthotopic tumors with ZIP4 over-expression or silencing.* The gene expression profile of PC orthotopic tumors from the xenografts with ZIP4 over-expression or silencing is performed by microarray analysis in order to additionally understand potential regulators and targets involved in ZIP4-mediated PC growth. Microarray experiment are done by using the comprehensive human Genome U133 PLUS 2.0 array chips (Affymetrix, Santa Clara, CA). The data is analyzed using computational signaling pathway analysis and selected genes of interest are validated using real time RT PCR and immunostaining for related proteins as described above.

[0340] *Zinc concentration detection.* Since ZIP4 is a specific dietary zinc transporter, it is important to determine whether the zinc levels in the above mentioned stable PC cell lines or tumor tissues collected from the nude mice correlate with the expression of ZIP4.

The zinc levels in human PC tissue specimens are also examined. Very few studies have been done to compare zinc profiles between cancer and surrounding normal tissues and body fluids (Taylor *et al.*, 2003; Letvin *et al.*, 1997; Costello *et al.*, 1999), and no such study has been done in PC patients. In one embodiment of the invention, over-expression of ZIP4 accumulates more zinc which promotes PC growth, and silencing of ZIP4 decreases the zinc accumulation which inhibits PC growth. In one embodiment of the invention, zinc levels may correlate with PC pathogenesis. Inductively coupled plasma mass spectrometry (ICPMS) is used to examine the zinc concentration in the stable BxPC-3, Capan-1, and Capan-2 cells which over-expressing ZIP4 or silencing ZIP4 as described previously (Li et al, 2007). 200 pancreatic tissues of adenocarcinoma are included along with PanINs at different stages. In addition, 10 normal pancreases from organ donors and autopsy cases, and 20 chronic pancreatitis specimens as above is studied. Briefly, cell pellets (about 10^4 cells each sample) are digested in 70% (vol/vol) HNO_3 at 70°C for 1 hour. For tissues, subcutaneous or orthotopic primary tumors of the same size from the nude mice are collected and homogenized. Homogenized mouse tissues (~50 mg) are placed in septum-sealed glass tubes and treated with 0.1 mL of 70% (vol/vol) HNO_3 overnight. All these samples are finally diluted with HPLC grade water to 2 ml for quantitative assay. Zinc concentration in cell lines and tissues is determined by ICPMS (ELAN 9000, PerkinElmer, Norwalk, CT) and normalized to the total protein content as described previously (Li *et al.*, 2007; Lowe *et al.*, 2004).

EXAMPLE 25

SUMMARY OF EXAMPLES 26-29

[0341] The following examples describe determination of whether over-expression of ZIP4 a) upregulates the transcription of IL-6 through CBP/CREB transcription factors, and b) affects PC cell proliferation and tumor growth via IL-6/STAT3 pathway.

[0342] It has been found that over-expression of ZIP4 significantly activated STAT3, and upregulated IL-6 and cyclin D1 levels in PC. STAT3 is activated in most PC cells at various levels. The downstream targets of STAT3 include genes that control survival, and cell proliferation, such as cyclins. Therefore, STAT3 and related molecules play critical roles in cancer pathogenesis and progression including PC (Scholz *et al.*, 2004). IL-6 is a major upstream activator for STAT3 (Bharadwaj *et al.*, 2007; Aaronson and Horvath, 2002; Horvath, 2000;

Howman *et al.*, 2000; Bowman *et al.*, 2000; Lang *et al.*, 2007). Previous studies have shown that IL-6 might also interact with zinc transport pathway by responding to plasma zinc concentration (Mariani *et al.*, 2007; Liuzzi *et al.*, 2005; Liuzzi *et al.*, 2006). IL-6 is controlled by the availability of zinc ion, whose intra-cellular transport is regulated by zinc transporters and metallothioneins (MTs), the zinc carrier proteins (Mariani *et al.*, 2007). Zinc also stimulated peripheral blood mononuclear cell (PBMC) to release many cytokines including IL-6 (Rink *et al.*, 2005; Driessen *et al.*, 1987; Salas and Kirchner, 1987; Scuderi, 1990). In the previous examples, a close correlation between ZIP4 and IL-6/STAT3 pathway in PC was found. Therefore, ZIP4 induced IL-6 upregulation represents a new mechanism to activate the downstream STAT3 pathway in PC. In one embodiment of the invention, ZIP4 over-expression causes increase of IL-6, which in turn activates STAT3, and leads to increased cyclin D1 expression, resulting in increased cell proliferation and tumor progression. The following examples determine how ZIP4 stimulates IL-6/STAT3 pathway, therefore promoting PC growth.

EXAMPLE 26

DETERMINATION OF ZIP4 CORRELATION TO EXPRESSION OF IL-6 IN HUMAN PANCREATIC CANCER

[0343] It has been shown that over-expression of ZIP4 caused upregulation of IL-6 in xenografts of MIA-ZIP4 cells, therefore, in one embodiment of the invention IL-6 correlates with ZIP4 expression levels in human PC tissues. Western blot analysis, immunohistochemistry staining, and Bio-Plex cytokine assay are performed to determine the expression of IL-6 in human PC tissue, cell lines and conditioned medium samples. 200 pancreatic adenocarcinoma tissues, 10 normal pancreases from organ donors and autopsy cases, and 20 chronic pancreatitis specimens are examined. The expression of IL-6 in stable PC cell lines is also examined.

[0344] *Immunohistochemistry and western blot.* Same samples of human PC as described in previous examples are studied in the following examples. Anti-IL-6 Ab (Cell Signaling Technology) which has been used in previous examples is used to detect the IL-6 proteins in the paraffin sections of human PC samples as described above. For Western blot analysis, proteins are extracted from the above FFPE tissue sections and probed with anti-IL-6 (1:2000) Abs followed by detection with enhanced ECL plus kit as above.

[0345] *Bio-Plex Cytokine Assay*. Plasma and serum samples from the above patients are also used to detect the concentration of IL-6 with Bio-Plex cytokine assay (Bio-Rad, CA) according to the manufacturer's protocol as described previously (Li *et al.*, 2006; Li *et al.*, 2007). Briefly, 50 µl of plasma or serum samples or cytokines standards are plated in a 96-well filter plate coated with IL-6 Ab and incubated overnight on a platform shaker at 300 rpm at room temperature. Data from the reaction is then acquired and analyzed using the Bio-Plex suspension array system (Luminex 100 system) from Bio-Rad Laboratories as described previously (Li *et al.*, 2006).

[0346] The expression of IL-6 in stable PC cell lines in which ZIP4 is over-expressed or silenced by shRNA is also examined. In one embodiment of the invention expression level of ZIP4 is correlated with the expression of IL-6 in human PC cells. Stable Capan-1, BxPC-3, and Capan-2 cells over-expressing ZIP4 gene or ZIP4 shRNA, and cells expressing vector controls are examined for the expression of IL-6 by western blot analysis. Briefly, cells are lysed and proteins are extracted and probed with anti-IL-6 (1:2000) Abs followed by detection with enhanced ECL plus kit as above. For IL-6 detection in conditioned medium, the supernatant of the cultured medium is collected after cells reach 60% confluence as described previously (Bharadwaj *et al.*, 2007), and 50 µl of the conditioned medium is plated in a 96-well filter plate coated with IL-6 Ab. The concentration of IL-6 is determined by the Bio-Plex suspension array system (Bio-Rad, CA) as above.

EXAMPLE 27

DETERMINATION OF IL-6 TRANSCRIPTION REGULATION OF ZIP4 THROUGH CBP/CREB

[0347] Both transcriptional and post-transcriptional regulation appear to be involved in IL-6 gene expression (Vanden Berghe *et al.*, 1998). The murine and human 400-bp 5'-flanking region of the IL-6 gene is highly conserved, and contains a number of *cis*-acting elements, which are required for gene induction by a variety of stimuli (Tanabe *et al.*, 1988). Response elements for at least four classes of transcription factors have been identified in this regulatory region upstream of IL-6 gene: cAMP response element-binding protein (CREB), activator protein-1 (AP-1), CCAAT/enhancer binding protein (C/EBP), and nuclear factor-κB

(NFκB) (Tanebe *et al.*, 1988, Akira *et al.*, 1990; Dendorfer *et al.*, 1994; Libermann and Baltimore, 1990). The promoter enhancing activity of CREB is dependent on the interaction with a zinc finger transcription factor CBP/p300 (CREB binding protein). CBP is the coactivator for CREB, and its activity is zinc dependent (Sahar *et al.*, 2007; Nyborg and Peersen, 2004). Therefore, in one embodiment of the invention over-expression of ZIP4 causes increased activity of CBP, which activates CREB and increases the transcription of IL-6 (FIG. 18).

[0348] *Electrophoretic mobility shift assay (EMSA).* In order to further show that ZIP4 increases DNA binding activity to cAMP response element, EMSA are performed with nuclear extracts from the stable Capan-1, BxPC-3, Capan-2, MIA PaCa-2, and ASPC-1 cells over-expressing ZIP4 gene or ZIP4 shRNA, and cells expressing vector controls, using a radioactively labeled 28 bp double-stranded oligonucleotide containing the cAMP response element 5'- AGA GAT TGC CTG ACG TCA GAC AGC TAG -3' (SEQ ID NO: 14) (Hershko *et al.*, 2002). The basic protein–DNA binding reaction mixture (12.5 ml) contains 18 mM HEPES (pH 7.9), 2 mM $MgCl_2$, 50 mM KCl, 10 mM DTT, 10% glycerol, 0.2 mg/ml BSA, 80 mg/ml deoxyinosine- and deoxycytosinenucleotide copolymer duplex (poly(dI-dC) z poly(dI-dc)), 15 fmol 32P-labeled probe, and 2 μg NE. The competition reaction is performed by adding 60-fold excess unlabeled double-stranded consensus oligonucleotide to the reaction mixture. Reaction mixtures are incubated at 25°C for 20 min and analyzed by 5% polyacrylamide gel electrophoresis (PAGE) and autoradiography as described previously (Otsuka *et al.*, 1994).

[0349] *Promoter activity assay.* For assessing CREB directed IL-6 promoter activity, a 2.1kb IL-6 promoter sequence is amplified by PCR using upstream primer at position -2,161 to -2,138 bp of the IL-6 gene start site: 5'-CTGAGCTCTCCAAGGCAGAGACTCT-3' (SEQ ID NO: 15) and down stream primer at position -41 to -62 bp: 5'-ACTCGAGATCATGGGAAAATCCCAC- 30 (SEQ ID NO: 16) (underlined sequences are the restriction enzyme sites for cloning the PCR products). The upstream primer contains a native restriction enzyme site for SacI and the down stream primer was designed with an XhoI enzyme site flanking the 5' end of primer sequences. After digestion with SacI and XhoI, the PCR product is cloned into a luciferase report vector, pGL3-promoter vector (Promega Corp.) between the SacI and XhoI sites. The resulted clone designated as pGL3-IL6-Luc-wt contains a 2,120 bp IL-6 promoter sequence from -2,161 to -41 bp upstream of the IL-6 gene. The pGL3-IL-6-CRE-m luciferase reporter plasmid containing point mutations on IL-6-cAMP response

element (5'-ATGCTAAAGG*TG*GTCACATT-3', (SEQ ID NO: 17) *TG* are mutations compared with AC in wild type), are generated by site directed PCR mutagenesis using pGL3-IL6-Luc-wt as a template as described previously (Li *et al.*, 2001). As controls, pGL3-IL-6-NFκB-m, pGL3-IL6-C/EBP-m, and pGL3-IL6-AP1-m luciferase reporters containing point mutations on IL6-NFκB, IL6-C/EBP, and IL6-AP1 transcription binding sites, respectively, by site directed PCR mutagenesis are also included (Xiao *et al.*, 2004). Transient transfection followed by assessing luciferase activity in above mentioned ZIP4 over-expressing or knocking down cells helps to confirm the increase or decrease of the IL-6 promoter activity respectively (Xiao *et al.*, 2004; Otsuka *et al.*, 1994). The pRL plasmid, a Renilla reniformis luciferase reporter vector, are co-transfected with all the samples as internal control and normalization of transfection efficiency.

EXAMPLE 28

DETERMINATION OF THE FUNCTIONAL RELATIONSHIP OF IL-6 AND ZIP4

[0350] Both ZIP4 and IL-6 have been shown to be involved in regulating cell proliferation, therefore, the cell proliferation in stable PC cells when treated with exogenous synthetic IL-6 or IL-6 neutralizing Ab and siRNA are examined. In one embodiment of the invention exogenous IL-6 treatment leads to activated STAT3 and increased cell proliferation, and blocking IL-6 with neutralizing Ab or siRNA causes decreased STAT3 activation, and decreased cell proliferation.

[0351] *Anchorage-dependent growth.* Stable MIA-ZIP4, Capan-ZIP4, and BxPC-ZIP4 cells which over-express ZIP4 and the vector control cells are seeded in 6-well plates. After reaching 50% confluence, the medium is replaced with the same growth medium containing 100ng/mL of IL-6 or 1 ug/mL of anti-IL-6 neutralizing Ab and the culture is continued for 24 h. For siRNA blocking, 100nM of IL-6 siRNA (5'-CCAGGAGAAGA TTCCAA AGATGTAGCCGC-3', Origene) is transfected into the cells with lipofectamine 2000 as described before (Li *et al.*, 2008). After 24 h, a portion of cell cultures are collected to confirm the STAT3 expression and phosphorylation status with Western blot analysis as described above, and the remaining cells are continuously cultured for functional assays as previously described (Bharadwaj *et al.*, 2007). $2x10^3$ cells per well are used for the proliferation assays (determined using a [^{3}H]thymidine incorporation and MTS assay) as above.

[0352] *Anchorage-independent growth (Colony formation assay).* Because the tumorigenic potential of adherent cancer cells is better reflected by anchorage-independent growth, soft agar assay is performed. The above mentioned stable cells are seeded in 6-well plates at 10^3 cells per well in triplicate as single cell suspension in Sea Plaque Agarose (Hoefer Scientific Instruments, San Francisco CA) and DMEM containing 10% FBS. The soft agar is overlayed on a 0.8% agarose layer containing DMEM and 10% FBS (n=3 wells/cell line). The plates is incubated for 2-3 weeks and colonies of more than 20 cells are counted.

EXAMPLE 29

DETERMINATION OF IL-6 DOWNSTREAM PATHWAY CONTRIBUTION TO PANCREATIC CANCER PATHOGENESIS AND PROGRESSION

[0353] Activation of the STAT3 signaling pathway plays an important role in the progression of PC and that blockade of STAT3 signals may provide a novel therapeutic strategy for PC (Toyonaga *et al.*, 2003). It has been found that over-expression of ZIP4 caused increased phosphorylation of STAT3 in MIA PaCa-2 cells. One of the STAT3 target gene cyclin D1 was also found to be correlated with the expression level of ZIP4 in PC, which indicates that STAT3/cyclin D1 pathway might be one of the major signaling pathways in ZIP4 mediated PC tumor growth. The previous examples and other studies also suggest that IL-6, an upstream activator of STAT3, might be an important intermediate signaling molecule connecting ZIP4 and STAT3 pathways. Therefore, ZIP4 induced IL-6 upregulation represents a new mechanism to activate the downstream STAT3 pathway in PC. It is determined whether blocking of STAT3, and cyclin D1 with chemical inhibitors or siRNAs leads to decreased cell proliferation, and cell cycle arrest, affects ZIP4 mediated tumor progression. Stable MIA PaCa-2, Capan-1, BxPC-3 cells which over-express ZIP4 are used, and STAT3, and cyclin D1 are individually blocked by chemical inhibitors, Abs, or siRNAs. The read out is cell proliferation and cell cycle progress.

[0354] *Cell proliferation assay.* For anchorage-dependent growth, stable MIA-ZIP4, Capan-ZIP4, and BxPC-ZIP4 cells which over-express ZIP4 and the vector control cells are seeded in 6-well plates. For chemical inhibitor and Ab blocking, after reaching 50% confluence, the medium is replaced with the same growth medium containing 100 μg/ml of tyrphostin AG490 (Calbiochem La Jolla, CA) or anti-cyclin D1 Ab (Cell Signaling), and the

culture is continued for 24 h. For siRNA blocking, cells are transfected with STAT3 (5'-GCTGACTA CACTGGCAGAGAAACTCTTGG-3'), (SEQ ID NO: 18) or cyclin D1 siRNAs (5'-TGGAACACCAGCTCCTGTGCTGCGAAGTG-3', Origene, SEQ ID NO: 19), and kept for 24 hrs. After 24 h, a portion of the above cell cultures are collected to confirm the STAT3 expression and phosphorylation status with Western blot analysis, and the remaining cells are continuously cultured for functional assays as previously described (Bharadwaj *et al.*, 2007). $2x10^3$ cells per well are used for the proliferation assays (determined using a [^{3}H]thymidine incorporation and MTS assay) as above. For anchorage-independent growth, the above mentioned stable cells are seeded in 6-well plates at 10^3 cells per well in triplicate as single cell suspension in Sea Plaque Agarose and DMEM containing 10% FBS as above. The plates are incubated for 2-3 weeks and colonies of more than 20 cells are counted.

[0355] *Cell cycle analysis.* To further elucidate the molecular mechanisms responsible for STAT3-regulated growth in PC cells, examination is made of the cell cycle progression which is a key component responsible for growth control. The above stable cells are synchronized by serum starvation for 24 hours, which results in G0/G1-phase arrest. Cell are then incubated with growth medium with 2% fetal bovine serum. Cells are collected at 4 and 8 h, permeabilized, stained with propidium iodide, and analyzed by flow cytometry (FACSCalibur, BD, San Jose, CA), and the cell cycle distribution is quantified by using Flowjo software (TreeStar Inc., Ashland, OR) (Li *et al.*, 2008).

EXAMPLE 30

STATISTICAL ANALYSIS OF EXAMPLES 26-29

[0356] Statistical analysis is performed with MiniTab statistical software as described above. For *in vitro* experiments, including cell proliferation, immunohistochemistry, cytokine assay, and real time RT PCR, data are expressed as mean ±SD, and 95% confidence intervals. After appropriate normalizing transformation, significant differences in cell proliferation and gene expression between control and experimental groups are determined by Student's *t* test (two-tail). All experiments are repeated at least three times with triplicates for each sample.

EXAMPLE 31

OVERVIEW OF EXAMPLES 32-35

[0357] The following examples determine whether a) 3 cycles of liposome/ZIP4 shRNA treatment, and b) combinational therapy of liposome/ZIP4 shRNA plus gemcitabine is effective on PC in 2 mouse models.

[0358] The novel finding of this invention is that dietary zinc transporter ZIP4 contributes to PC growth and therefore serves as a new molecular target for ZIP4-based therapy in PC. Previous examples showed that silencing of ZIP4 significantly inhibited PC tumor growth and increased survival rate in nude mice xenograft model. The previous examples also showed that the ZIP4 expression levels are reduced after ZIP4 shRNA therapy, which could affect the response of the PC cells to subsequent therapy. By defining the ZIP4 profile of PC cells before and after therapy, the ZIP4 profile can be used to guide the choice of initial and sequential ZIP4-based therapies. Mouse with high levels of ZIP4 may respond to the shRNA therapy better than the mouse with low level of ZIP4. Liposome delivery of ZIP4 shRNA is used, and the effectiveness of the delivery system depends on the ZIP4 profile. The above examples indicates that by wrapping the shRNA in liposomes, multiple cycles of shRNA treatment is feasible and are more effective than a single cycle treatment (Liu *et al.*, 2008). This concept is important, since it may require multiple treatment cycles to have an impact on survival in patients with metastatic PC and that sequential therapy could be guided by the ZIP4 profile in the mouse model. The efficacy of shRNA therapy in a Kras transgenic mouse model is tested.

[0359] To increase the efficacy of shRNA therapy, in one embodiment of the invention combinational therapy is used (double or triple therapy). The combinational therapies with ZIP4 shRNA and gemcitabine is tested in both xenograft mouse model and the Kras transgenic mouse model. Furthermore, low zinc diet is also included in the combinational therapy as an adjuvant treatment. Low zinc diet might lower the risk of PC, which provides dietary recommendations on PC patients by increasing the uptake of vegetable and fruit and reducing the uptake of meat. The Kras transgenic mouse model tests this, by feeding the animals with diets containing different ratio of zinc besides the double therapy mentioned above.

EXAMPLE 32

DETERMINATION OF THE THERAPEUTIC EFFECT OF ZIP4 SHRNA

[0360] Liposomal wrapped human ZIP4 shRNA is prepared as described previously. The effects of ZIP4 shRNA on cell proliferation in vitro (cell culture model) and tumor progression in vivo (xenograft animal model and transgenic mouse model) is investigated. ASPC-1 and Capan-2 cells are used because of the high endogenous levels of ZIP4 expression in these two cells.

[0361] *Cell culture model.* ASPC-1 and Capan-2 cells are seeded in 96-well plates (2×10^3 cells/well), and serum starved for 24 hrs. Cells are treated with chemically synthesized ZIP4 siRNA: 5'ACGTAGC ACTCTGCGACATGGTCAGGATG3' (SEQ ID NO: 9) (custom synthesized from Ambion, Austin, TX) at different concentrations (0, 10, 20, 50, and 100 nM) for different times (24, 48, and 72 hrs) by using Amine, a commonly used transfection reagent (Ambion) following standard protocol. In a separate setting, cells are transfected by ZIP4 shRNA in pSuper vector (Clontech, CA). Cell proliferation is determined by MTS assay and [^{3}H]thymidine incorporation assay as described above. All samples are triplicated, and all experiments are repeated at least three times.

[0362] *Xenograft animal model.* The efficiency of ZIP4 shRNA in vivo by treating preexisting pancreatic cancer xenografts with ZIP4 shRNA is tested. To increase the stability of the RNA molecules in vivo, liposome are used to deliver the ZIP4 shRNA to the preexisting pancreatic cancer xenografts. In the subcutaneous (s.c.) model, 3×10^6 ASPC-1 or Capan-2 cells are injected to the right flank of 6-8 weeks old male nude mice (n=40). Treatment starts 2 weeks after tumor cell implantation, when the tumors reached the approximate size of 5 mm in diameter. Liposome/ZIP4 shRNA (30 μg) or liposome/scrambled shRNA are injected intratumorally 3 times with two weeks apart, and tumor size is measured weekly. The tumor volume is determined by the formula: tumor volume [mm^3] = (length [mm]) x (width [mm])^2 x 0.52. To further study the effect of ZIP4 shRNA in metastatic pancreatic cancer, an orthotopic model of pancreatic cancer is used, which has been well developed. 3×10^6 ASPC-1 or Capan-2 cells are injected into the pancreas of the nude mice (n=40), and treatment starts 1 week later. The mice are injected intravenously (i.v.) with liposome/ZIP4 shRNA complex (30 μg) or liposome/scrambled shRNA 3 times with 1 week apart. For both s.c. and orthotopic model, five mice are sacrificed before each cycle of treatment to determine the presence and size of tumors, which are harvested for ZIP4 profile studies and pathological analysis as described above. Survival is analyzed, as well as any toxicity. On day 42 after tumor cell inoculation, all surviving

mice are euthanized by CO_2 exposure and evaluated macroscopically for the presence of orthotopic tumor and the metastases in the abdominal cavity. All tumor tissues are collected and evaluated using H&E staining, immunohistochemistry and Western Blot for ZIP4 protein expression, Sanger Sequencing of DNA mutation (genetic variation), and microarray analysis for RNA to determine the effect of each cycle of treatment on the ZIP4 profile and other gene alterations. Zinc concentration in the tumor tissues are examined by ICPMS as described above.

[0363] *Transgenic animal model.* These studies are also repeated in MDA Kras transgenic mice to determine whether 3 cycles of liposome/ZIP4 shRNA treatment inhibits growth of endogenous PC in immune competent mice. 30% of the Kras transgenic mice develop PC at 4 to 6 months, and 50% develop PC at 9 months. Therefore, the ZIP4 shRNA treatment is started at 7 months when about 40% of the mice have PC. Same dose of iv liposome/mouse ZIP4 shRNA (5'TCTGTGGAGAATGTCTTGGCTCTAG GCAA3') (SEQ ID NO: 20) complex (30 μg) or liposome/scrambled shRNA is injected into the Kras transgenic mice (n=50) 3 times with 2 weeks apart. Five mice are sacrificed before each cycle of treatment to determine the presence and size of tumors, which is harvested for ZIP4 profile studies and pathological analysis as described above. Survival is analyzed, as well as any toxicity. Two weeks after the final treatment, all surviving mice are euthanized. Detailed analysis of DNA, RNA, protein, and zinc concentration are performed as above.

[0364] These studies may be repeated using MIA PaCa-2 and BxPC-3 cells to determine if ZIP4 shRNA inhibits growth of human PC cells with low ZIP4 expression levels.

EXAMPLE 33

DETERMINATION OF THE EFFICACY OF COMBINATORIAL THERAPY OF PANCREATIC CANCER

[0365] The most exciting data from the previous examples indicated that silencing ZIP4 by shRNA significantly inhibited PC tumor progress and increased survival rate of the animal with xenografts. These results show that ZIP4 based shRNA therapy can buy time to treat the PC patients, if combined with other therapies such as chemotherapy (double or triple therapy), further increases the survival rate of PC patients. Since shRNA therapy inhibits tumor cell growth through mechanisms different from standard chemotherapies, it is possible that these

two therapies may synergize through their reciprocal enhancement of tumor cell killing. shRNA therapy may sensitize the tumor cells to chemotherapy.

[0366] Another adjuvant therapy as part of the combinational therapy is the use of low zinc diet. Previous epidemiology studies indicate an important role of low zinc containing food as part of a healthy diet for PC patient. Low zinc diet as a nutrition and dietary prevention may also help to slow down the tumor progression. The combination of ZIP4 shRNA therapy, chemotherapy, and/or low zinc diet treatment determineds if the combination enhances the antitumor effect of individual therapy.

[0367] *In vitro experiment.* Determination is made if there is any synergy between gemcitabine, a commonly used drug for PC chemotherapy, ZIP4 shRNA, and low zinc treatment in inhibiting PC cell proliferation. ASPC-1 or Capan-2 cells which have high endogenous ZIP4 expression are seeded in 6-well plates, and cells are treated with: 1) ZIP4 shRNA in the presence of gemcitabine at different concentrations (10^{-3}–10^{2} μg/ml); 2) ZIP4 shRNA with TPEN chelation; 3) TPEN chelation in the presence of gemcitabine at different concentrations (10^{-3}–10^{2} μg/ml); and 4) ZIP4 shRNA and TPEN chelation in the presence of gemcitabine at different concentrations (10^{-3}–10^{2} μg/ml). Cell viability is determined by MTS assay and apoptosis is determined by TUNEL staining as described in the above examples and previous publications.

[0368] *In vivo xenograft animal model.* Metastatic PC xenografts (orthotopic model) using ASPC-1 or Capan-2 cells are established as above. Mice are randomly divided into 7 groups (n=25) and are given following iv injections: 1) PBS; 2) once-daily injection of gemcitabine at a dose of 0.5 mg/mouse at every 3rd day; 3) once-daily injection of liposome/ZIP4 shRNA (30 μg) on every 7th day for 4 weeks; 4) once-daily injection of liposome/ZIP4 shRNA (30 μg) on every 7th day plus once-daily injection of gemcitabine on every 3rd day for 4 weeks; 5) once-daily injection of gemcitabine (0.5 mg/mouse) at every 3rd day fed with low zinc diet (3mg/kg diet, LZ); 6) once-daily injection of liposome/ZIP4 shRNA (30 μg) on every 7th day fed with low zinc diet (3mg/kg diet, LZ); 7) once-daily injection of liposome/ZIP4 shRNA (30 μg) on every 7th day plus once-daily injection of gemcitabine (0.5 mg/mouse) on every 3rd day for 4 weeks fed with low zinc diet (3mg/kg diet, LZ); Five mice from each group are sacrificed at days 7, 14, and 21 after treatment, and tumor tissues are harvested for ZIP4 profile studies and pathological analysis as above. Survival is analyzed, as well as any toxicity. On week 4 after initial treatments, all surviving mice are euthanized by CO_2 exposure and evaluated

macroscopically for the presence of orthotopic tumor and the metastases in the abdominal cavity. All tumor tissues are collected and evaluated using H&E staining, immunohistochemistry and Western Blot for ZIP4 protein expression, Sanger Sequencing of DNA mutation (genetic variation), and microarray analysis for RNA to determine the effect of each cycle of combinational treatment on the ZIP4 profile and other gene alterations. Zinc concentration in the tumor tissues are examined by ICPMS as described above.

[0369] *Transgenic animal model.* The effect of combinational therapy in the MDA Kras transgenic mice with various combinations as above is also investigated. Those mice are fed with modified AIN93G diet with low zinc (3mg/kg diet, LZ) and adequate zinc (30mg/kg diet, AZ), respectively, since 3 months after birth. At 7 months, mice from each diet group are randomly divided into 2 groups (n=50) and are given following iv injections: 1) once-daily injection of gemcitabine at a dose of 0.5 mg/mouse at every 3rd day; 2) once-daily injection of liposome/mouse ZIP4 shRNA (30 μg) on every 14th day plus once-daily injection of gemcitabine (0.5 mg/mouse) on every 3rd day for 6 weeks. Five mice from each treatment group are sacrificed every week, the presence of PanINs and PC in the mouse pancreas are evaluated by H&E staining of the collected tissues. The tissues are tested for ZIP4 profile studies and pathological analysis as described above. Two weeks after the final treatment, all surviving mice are euthanized. Detailed analysis of DNA, RNA, protein, and zinc concentration are performed as above.

EXAMPLE 34

STATISTICAL ANALYSIS FOR EXAMPLES 32-33

[0370] Statistical analysis is performed with MiniTab statistical software as described above. For *in vitro* experiments, including cell proliferation, and real time RT PCR, data are expressed as mean ±SD, and 95% confidence intervals. After appropriate normalizing transformation, significant differences in cell proliferation and gene expression between control and ZIP4 shRNA treatment group are determined by Student's *t* test (two-tail).

[0371] For *in vivo* experiments, calculated tumor incidence, and exact binomial confidence intervals (95%) are presented within individual experimental groups, and compared by Fisher's exact test. In animals that grow tumors, tumor volumes (or an appropriate

transformation, such as logarithm) are expressed as mean ±SD and 95% confidence intervals. Comparison of tumor volume among different groups is analyzed with single factor. Analysis of Variance (ANOVA) test. A *P* value <0.05 is considered statistically significant. Sample size for animal studies are determined by using nQuery Advisor. The number of mice proposed based on previous variability seen in tumor weight and a desired power and previous data (1-β) of 0.80 (Li *et al.*, 2007; Li *et al.*, 2008) as described above.

EXAMPLE 35

OTHER EXEMPLARY STUDIES

[0372] In one embodiment of the invention, 3 cycles of iv liposome/ZIP4 shRNA is used to treat ZIP4 low expressing cells such MIA PaCa-2 cells. In one embodiment of the invention, sequential cycles of liposome/ZIP4 shRNA is considered to enhance the therapeutic effect. In another embodimet of the invention, nanoparticle or adenovirus are used as alternative delivery systems. Nanoparticle delivery delivery can be used (Chono et al., 2008, Yoshizawa et al., 2008). Adenovirus delivery can also be used (Kargiotis et al., 2008, Yoo et al., 2008). In one embodiment of the invention, the combinational therapy is further enhance the anti-tumor activity of individual therapy. In another embodiment of the invention, low zinc diet feeding help to slow down the tumor progression of PC in the transgenic animal model. One of skill in the art realizes that multiple adminstration schemes may be used and adjusted to the individuals response. In one embodiment of the invention Gemcitabine is administered 3 days prior to shRNA therapy. This may sensitize tumor cells before the start of shRNA therapy.

EXAMPLE 36

PROTECTION OF SUBJECTS

[0373] All examples are exemplary in nature and one of skill in the art realizes that there are many other ways to practice the invention. Patients from any age, gender, religious affiliation, and race are recruited for the studies. Every effort is made to assure appropriate representation of all groups afflicted by pancreatic cancer. The ethnic and gender makeup of the population is anticipated to be similar to the demographics of present databases: 76% Caucasian, 12% Hispanic (mostly of Mexican origin), and 12% African American with 47% females and

53% males. The patient information collected includes demographics, exposure, family history, symptoms and physical findings at presentation, laboratory values, diagnostic imaging test results, details of the surgical treatment, histology from preoperative and operative specimen, pathologic staging data, details of chemotherapy, and radiation treatment, response to treatment in terms of follow-up imaging, disease-free survival, and overall survival, and quality of life survey data. The data are entered and stored in a password protected, HIPPA-compliant web-based database (Velos). To assure patient confidentiality, the specimen is logged into the database, names and specific identifications are removed and a 9-digit bar code is assigned to each specimen as they are deposited in the tissue bank. 200 PC specimens from operative cases and matching patient blood samples from the Elkins Pancreas Center are included in the study. The Elkins Pancreas Center tissue bank presently contains 197 PC specimens, and a complete matching set of surrounding normal pancreas, plasma, serum, and blood cells from the same patient. Samples are currently accruing at a rate of 4 patients per month. These studies are repeated in an independent cohort of PC with additional 100 PC specimens. The same rules apply to this cohort. In this study the potential of ZIP4 as a biomarker in human tissue microarrays derived from pancreatectomy specimens is tested using both recurrence and pancreas cancer specific mortality as endpoints. Data about the population of patients described above have been accrued using the Elkins Pancreas Center forms. The clinical and pathologic data of patients who meet the entry criteria is available for analysis with patient identifiers removed.

[0374] The studies are carried out in male athymic nude mice Nu/nu and Kras transgenic mice. All nude mice are 6 to 8 wk of age at the initiation of the experiments. Approximately 1,500 nude mice and 300 Kras transgenic mice are used in this proposal. To obtain statistically significant comparisons in mice, 30 mice per test group are used with 30 mice for controls per route. The numbers of mice used are clearly delineated and have been determined to be the minimal number to complete the studies. For details, please refer to statistical analysis plan.

[0375] The rationale to use nude mice is that they are immune-compromised and serve as a host for human PC cells. They reproduce well and are a hearty breed. These mice enable the study of the effect of ZIP over-expression and silencing in PC growth in vivo. Due to the need to determine basic information on the tumor growth and progression, mice have been

selected based on their availability, cost and the extensive knowledge about the intricacies of their immune system.

[0376] For all groups, parental tumor cells, GFP control tumor cells, or stable cell lines with shRNA or gene over-expression ($1\text{-}3\times10^6$ in 0.1 mL of PBS) are injected into the nude mice. For s.c. model, cells are injected into the right flank of the nude mice. A total of 30 mice per group are used. One week after the injection and every week thereafter, the length and width of the tumors that developed are measured. Mice are sacrificed at 90 days and tumor mass and volume are recorded. Volume is calculated as length $\times$ width$^2 \times 0.52$. For orthotopic model, mice are anesthetized with 2.5% avertin and a 0.5-1 cm incision is made in the left subcostal region. The tumor cells ($1\text{-}3\times10^6$) in a volume of 50 μL are injected into the body of the pancreas. The peritoneum and skin are closed with a 4.0 surgical suture. After 4 weeks, all surviving mice are euthanized by an overdose of CO_2 exposure and evaluated macroscopically for the presence of orthotopic tumors and the metastasis in the abdominal cavity. The orthotopic and metastatic tumor nodules are then explanted, counted and measured. For both s.c. and orthotopic experiments, the animals are euthanized when their tumor size reach 2 cm in diameter or the animals become moribund during the observation period, and the time of euthanization is recorded as the time of mortality. Procedures are used to avoid unnecessary discomfort, pain, or injury to the animals. Anesthetics is employed whenever appropriate. For shRNA and combinational therapy, nude mice and transgenic mice are iv injected with different treatment.

EXAMPLE 37

GENOMIC VARIATION OF ZIP4 IN HUMAN PANCREATIC CANCER TISSUES

[0377] The genomic variations (genomic profiling) of selected genes in DNAs isolated from more than 100 PC specimens were studied using sequencing technology. A number of mutations/SNPs in a few genes were found including Kras and somatostatin receptor 5 (SSTR5). The DNA has been extracted from PC specimens, and the whole genome was amplified. A panel of candidate genes was selected based on the known mutations and aberrant expressions in PC. Those genes were each PCR amplified and sequenced using Sanger sequencing and the results were analyzed with 2 different software programs developed at the HGSC for high-throughput sequence alignment and SNP calling. Validation of the selected mutations was done by using TaqMan genotyping on individual samples. Inherited and acquired

somatic mutations were differentiated by analyzing the DNA extracted from the blood of each patient or surrounding normal pancreas tissues with the tumor DNA. A total of 14 sequencing runs with an average of 30,000,000 bases sequenced per run were performed. Non-coding and synonymous mutations were identified in PC for all genes. To date, a total of 29 non synonymous mutations were identified in 9 genes. Non synonymous mutations, such as the known *K-RAS* mutations in codon 12 and 13 in PC, were part of the identified mutations which was expected. Specific PC mutations including mutations in *K-RAS*, *SSTR5*, and tumor protein p53 (*TP53*) were also found. To date somatic mutations were confirmed in *K-RAS* and *SSTR5* using TaqMan genotyping. Mutations were identified in the highly stroma-infiltrated PC without the need for tumor cell enrichment such as laser capture microdissection. The primers for ZIP4 gene (*SLC39A4*) have been designed and synthesized. The analysis of ZIP4 genetic variations in the PC samples from a database is underway. This data demonstrate the feasibility to define ZIP4 genetic variation using sequencing technique in a larger group of PC specimens.

[0378] Although the present invention and its advantages have been described in detail, it should be understood that various changes, substitutions and alterations can be made herein without departing from the spirit and scope of the invention as defined by the appended claims. Moreover, the scope of the present application is not intended to be limited to the particular embodiments of the process, machine, manufacture, composition of matter, means, methods and steps described in the specification. As one of ordinary skill in the art will readily appreciate from the disclosure of the present invention, processes, machines, manufacture, compositions of matter, means, methods, or steps, presently existing or later to be developed that perform substantially the same function or achieve substantially the same result as the corresponding embodiments described herein may be utilized according to the present invention. Accordingly, the appended claims are intended to include within their scope such processes, machines, manufacture, compositions of matter, means, methods, or steps.

REFERENCES

[0379] All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

PATENTS AND PATENT APPLICATIONS

U.S. Patent 5,446,137
U.S. Patent 5,714,606
U.S. Patent 5,614,617
U.S. Patent 5,652,099
U.S. Patent 5,672,697
U.S. Patent 5,670,663
U.S. Patent 5,700,922
U.S. Patent 5,708,154
U.S. Patent 5,763,167
U.S. Patent 5,792,847
U.S. Patent 5,859,221
U.S. Patent 5,872,232
U.S. Patent 5,886,165
U.S. Patent 5,466,786
U.S. Patent 5,223,618
U.S. Patent 5,470,967
U.S. Patent 5,378,825
U.S. Patent 5,777,092
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CLAIMS

What is claimed is:

1. A method of inhibiting proliferation of at least one cancer cell in an individual, comprising delivering to the individual an effective amount of a composition comprising an agent that inhibits ZIP4.

2. The method of claim 1, wherein the agent comprises nucleic acid.

3. The method of claim 2, wherein the nucleic acid comprises RNA.

4. The method of claim 3, wherein the RNA is further defined as siRNA.

5. The method of claim 4, wherein the siRNA is shRNA.

6. The method of claim 5, wherein the shRNA comprises SEQ ID NO: 7, SEQ ID NO: 8, and SEQ ID NO: 9.

7. The method of claim 6, wherein the shRNA is SEQ ID NO: 9.

8. The method of claim 1, wherein the cancer cell is a breast cancer cell, a prostate cancer cell, a lung cancer cell, or a pancreatic cancer cell.

9. The method of claim 8, wherein the cancer cell is a pancreatic cancer cell.

10. The method of claim 1, wherein the individual is further provided one or more additional anti-cancer therapies.

11. The method of claim 10, wherein the additional anti-cancer therapy comprises chemotherapy, radiotherapy, immunotherapy, gene therapy, surgery, or a combination thereof.

12. The method of claim 1, wherein the agent is delivered in multiple cycles of treatment.

13. The method of claim 1, wherein delivery is by liposomal delivery.

14. The method of claim 1, wherein the delivery is by viral delivery.

15. A method of treating an individual with cancer, comprising delivering to the individual an effective amount of a composition comprising an agent that inhibits ZIP4.

16. A kit for cancer treatment, said kit housed in a suitable container and comprising:

a first anti-cancer agent that inhibits ZIP4 expression and/or activity.

17. The kit of claim 16, wherein said first agent comprises siRNA.

18. The kit of claim 17, wherein said siRNA comprises SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, or any combination thereof.

19. The kit of claim 17, wherein said siRNA comprises shRNA.

20. The kit of claim 16, additionally comprising one or more anti-cancer agents.

21. The kit of claim 16, wherein one of said additional anti-cancer agent is a chemotherapeutic agents.

22. The kit of claim 21, wherein the chemotherapeutic agent is effective against breast cancer, is effective against lung cancer, is effective against prostate cancer, or is effective against pancreatic cancer.

23. The kit of claim 22, wherein said chemotherapeutic agent effective against pancreatic cancer comprises gemcitabine, 5-fluorouracil, cisplatin, irinotecan, paclitaxel, capecitabine, oxaliplatin, streptozocin, or a combination thereof.

24. The kit of claim 20, wherein one or more additional anti-cancer agent comprises one or more radioisotopes.

25. A method of screening for an agent that inhibits ZIP4, comprising the steps of:

(a) providing a candidate modulator;

(b) admixing the candidate modulator with a respective ZIP4 polynucleotide or polypeptide; and

(c) assaying association of the candidate modulator with the respective polynucleotide or polypeptide,

wherein when the candidate modulator associates with the respective polynucleotide or polypeptide, said candidate modulator is the agent.

26. The method of claim 26, wherein said association is further defined as binding.

27. The method of claim 26, further comprising manufacturing the agent.

28. The method of claim 26, further comprising delivering the agent to an individual that has cancer or that is suspected of having cancer.

29. A method of identifying a cancer in an individual comprising detecting ZIP4 in cells of the individual.

30. The method of claim 29, wherein other cancer markers are also overexpressed in the individual.

31. The method of claim 29, wherein the ZIP4 is ZIP4 protein, ZIP4 mRNA, or a combination thereof.

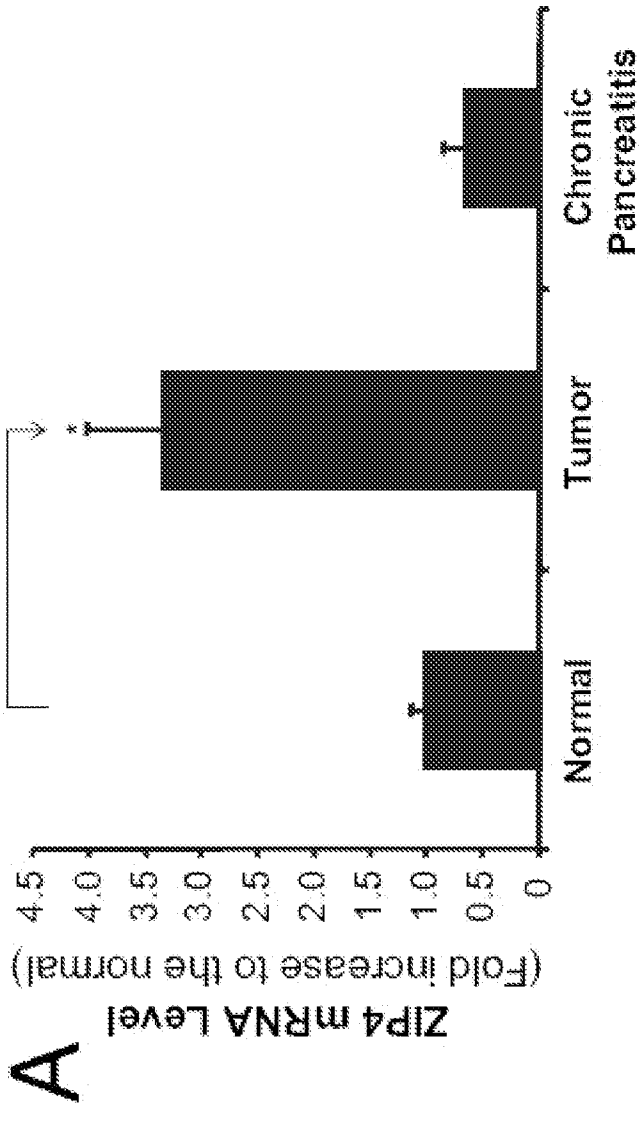
A
ZIP4 mRNA Level
(Fold increase to the normal)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Normal
Tumor
Chronic Pancreatitis

FIG. 1A

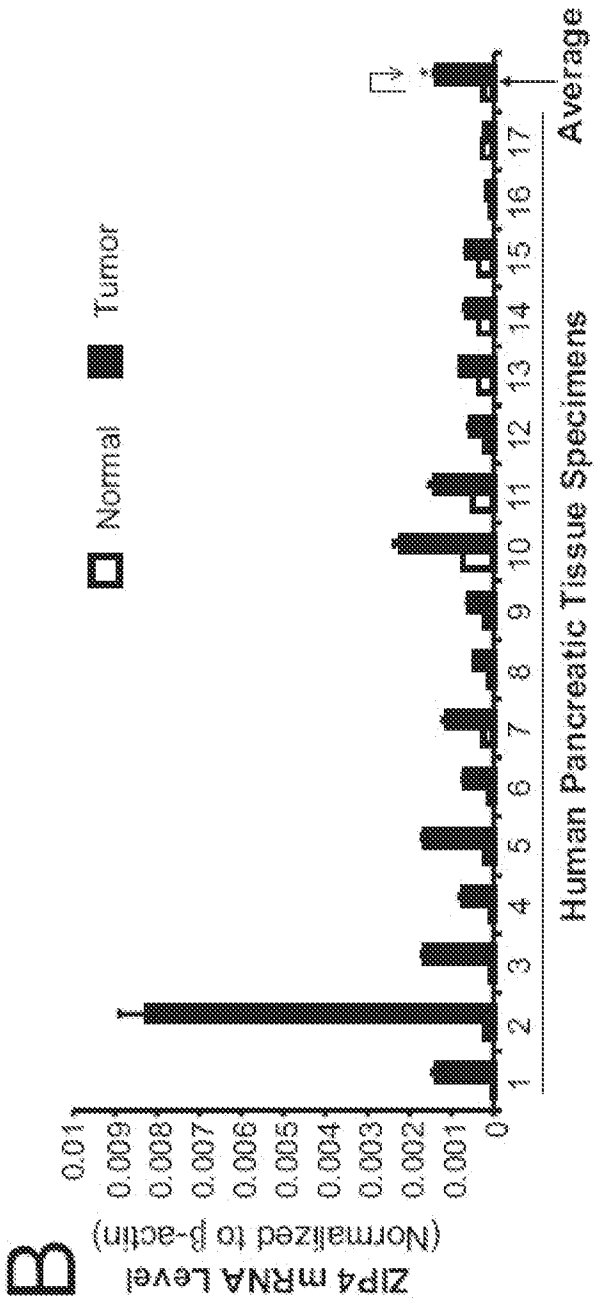
B
ZIP4 mRNA Level
(Normalized to β-actin)
0.01
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
Normal
Tumor
*
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Average
Human Pancreatic Tissue Specimens

FIG. 1B

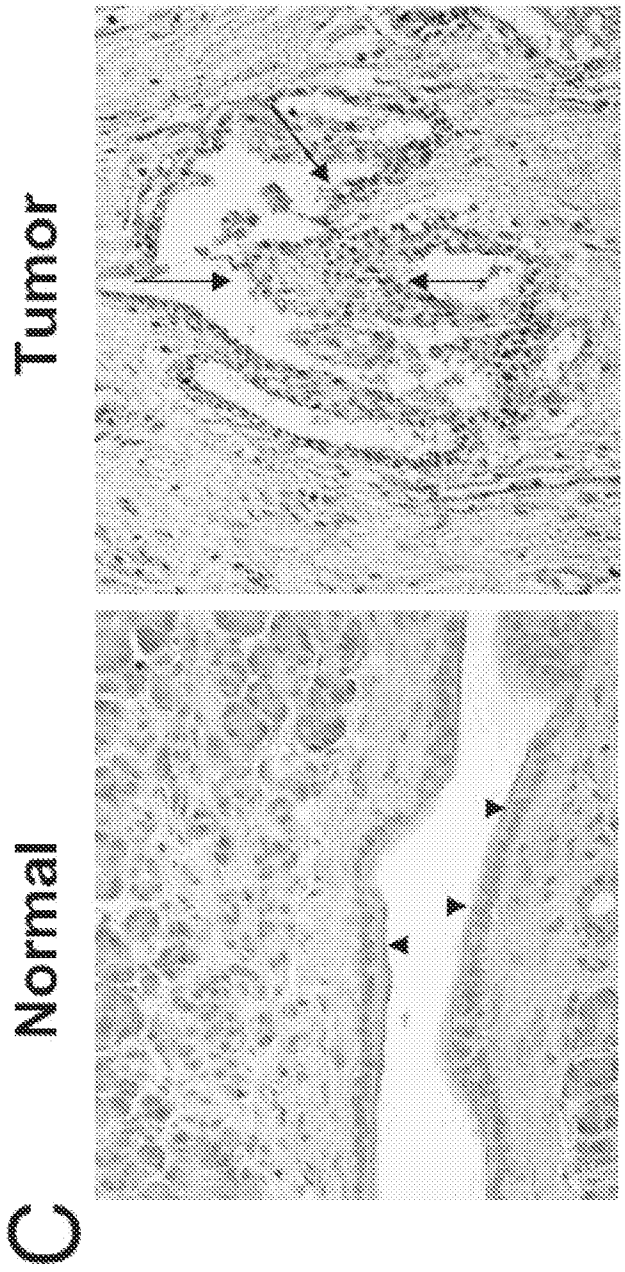

FIG. 1C

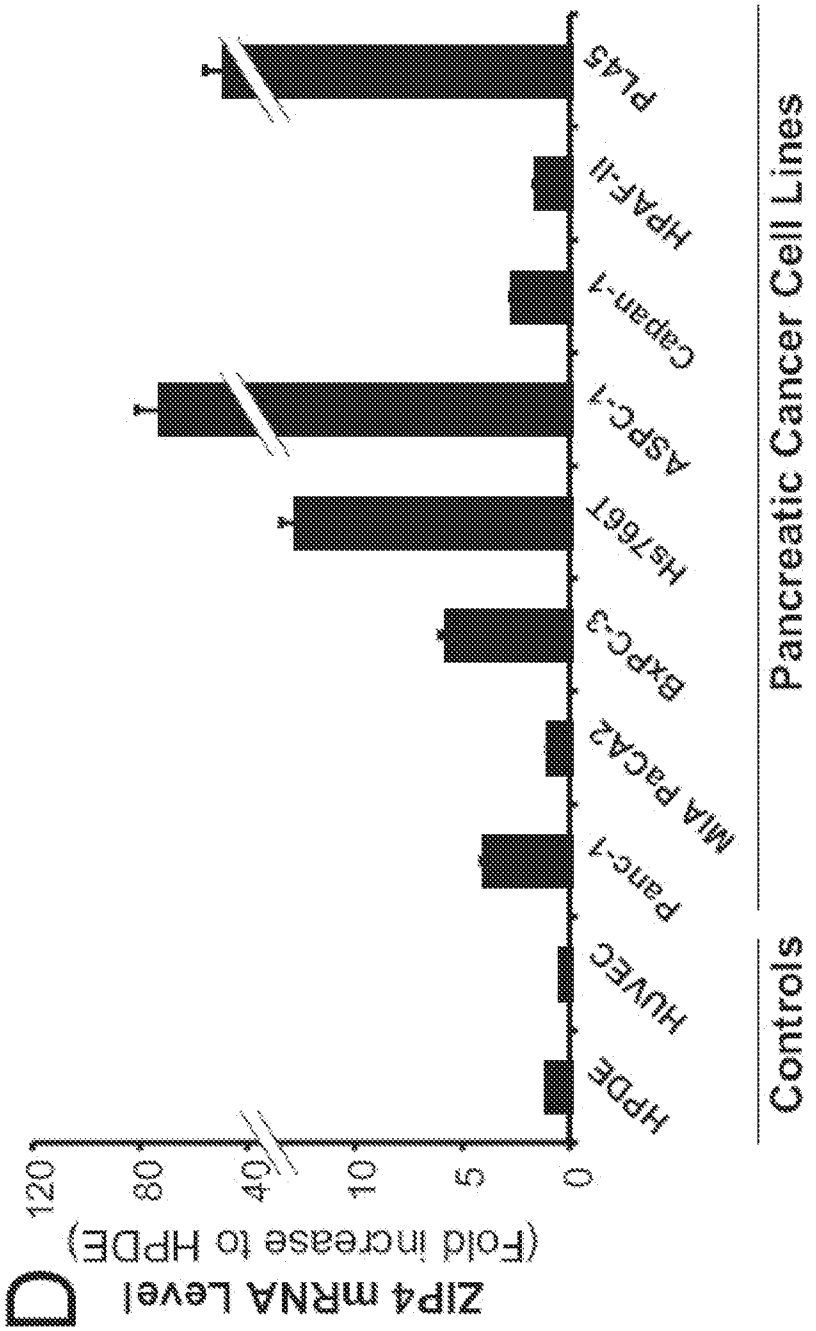
D
ZIP4 mRNA Level
(Fold increase to HPDE)
120
80
40
10
5
0
HPDE
HUVEC
Panc-1
MIA PaCA2
BxPC-3
Hs766T
ASPC-1
Capan-1
HPAF-II
PL45
Controls
Pancreatic Cancer Cell Lines

FIG. 1D

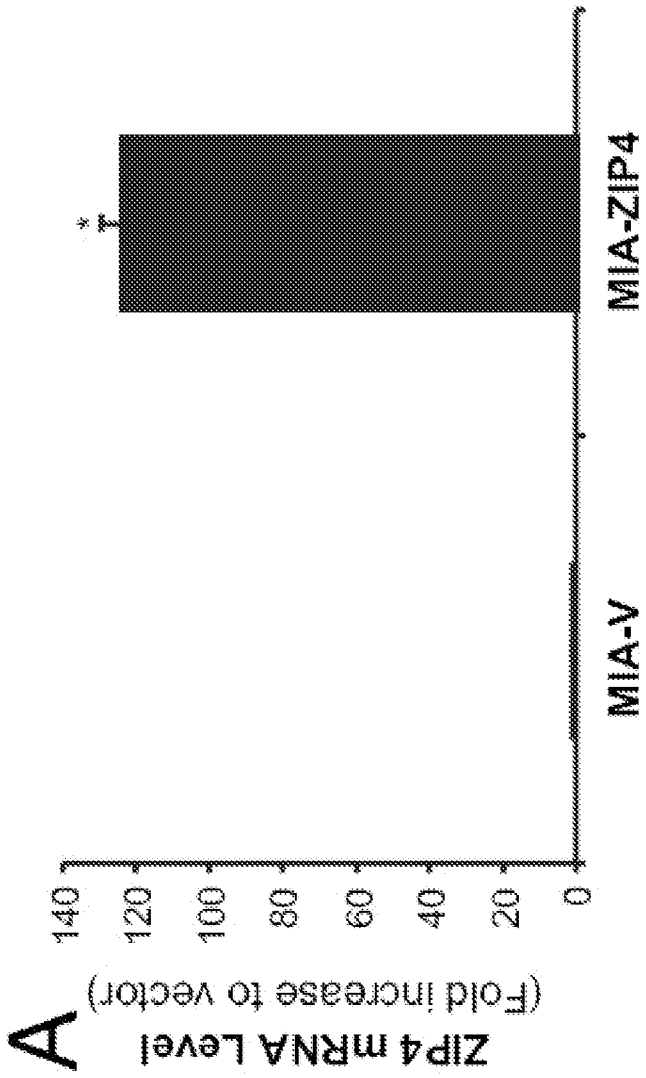
A
ZIP4 mRNA Level
(Fold increase to vector)
140
120
100
80
60
40
20
0
*
MIA-V
MIA-ZIP4

FIG. 2A

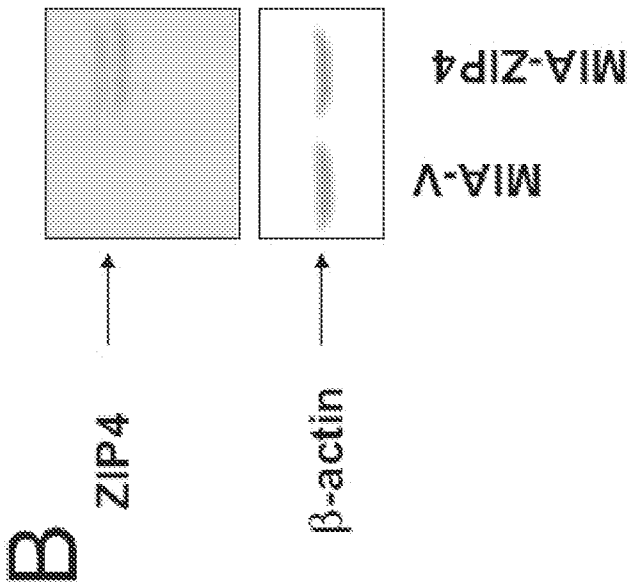

FIG. 2B

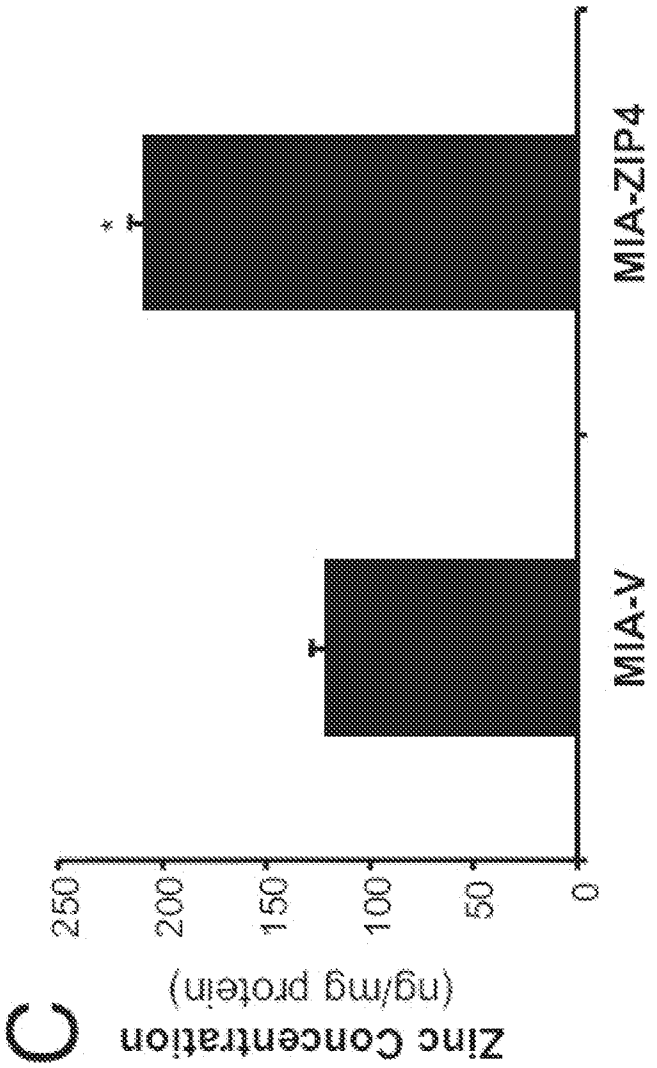
C
Zinc Concentration
(ng/mg protein)
250
200
150
100
50
0
MIA-V
MIA-ZIP4
*

FIG. 2C

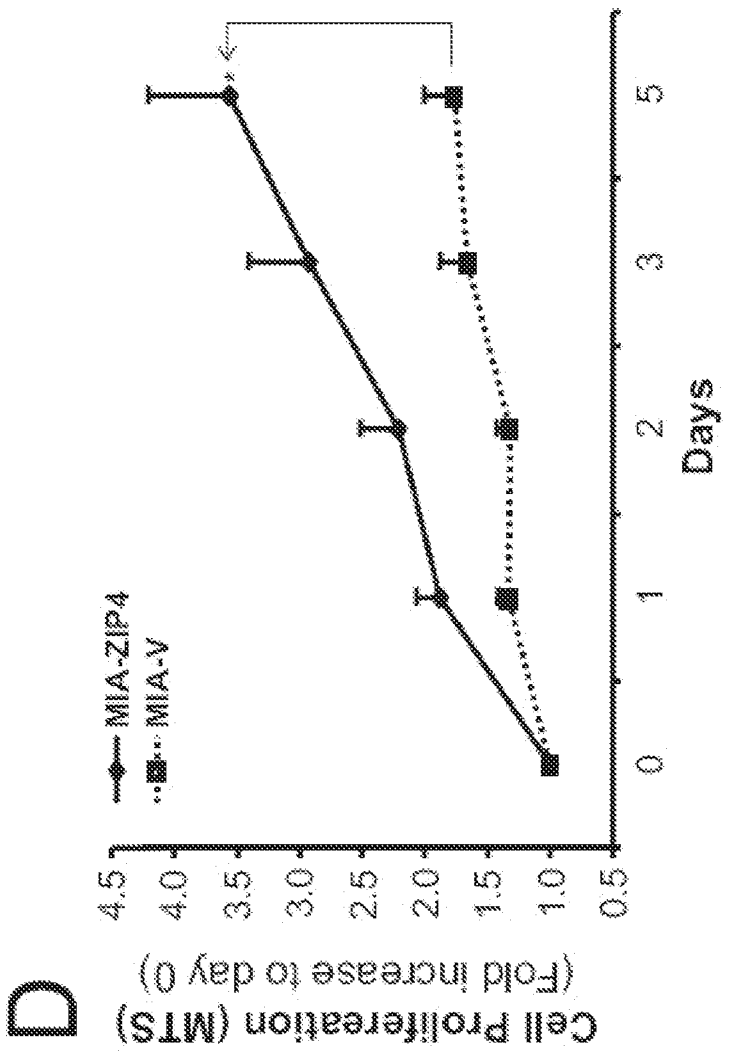
D
Cell Proliferation (MTS)
(Fold increase to day 0)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
1
2
3
5
Days
MIA-ZIP4
MIA-V

FIG. 2D

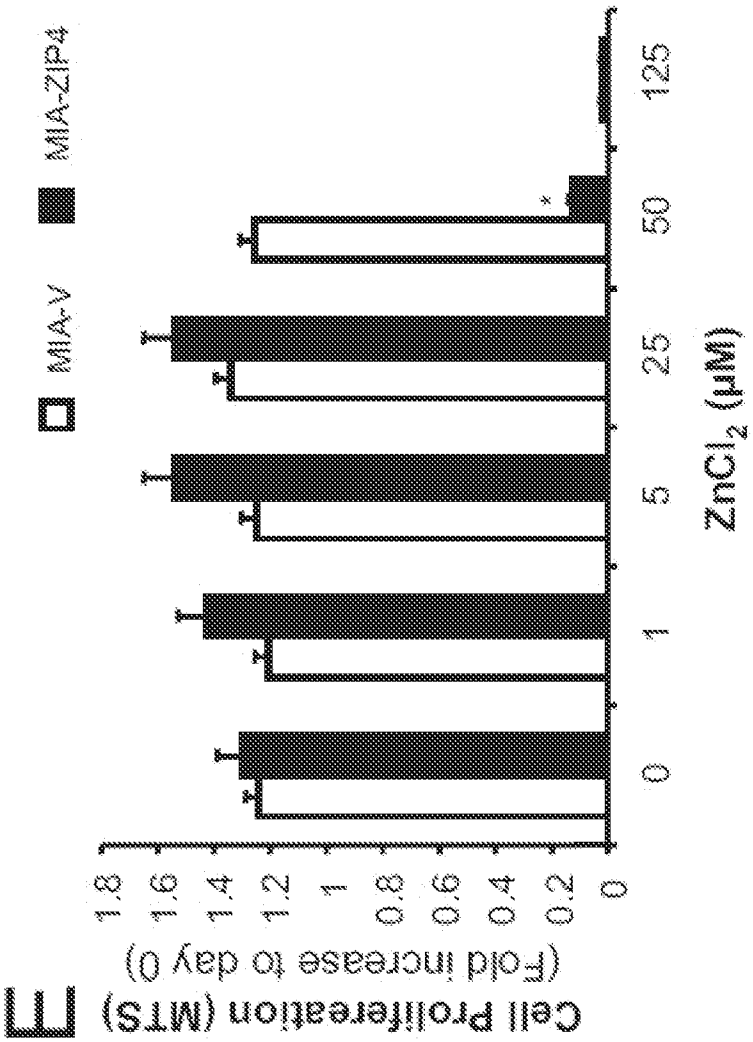
Cell Proliferation (MTS)
(Fold increase to day 0)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
MIA-V
MIA-ZIP4
0
1
5
25
50
125
ZnCl2 (μM)
*

FIG. 2E

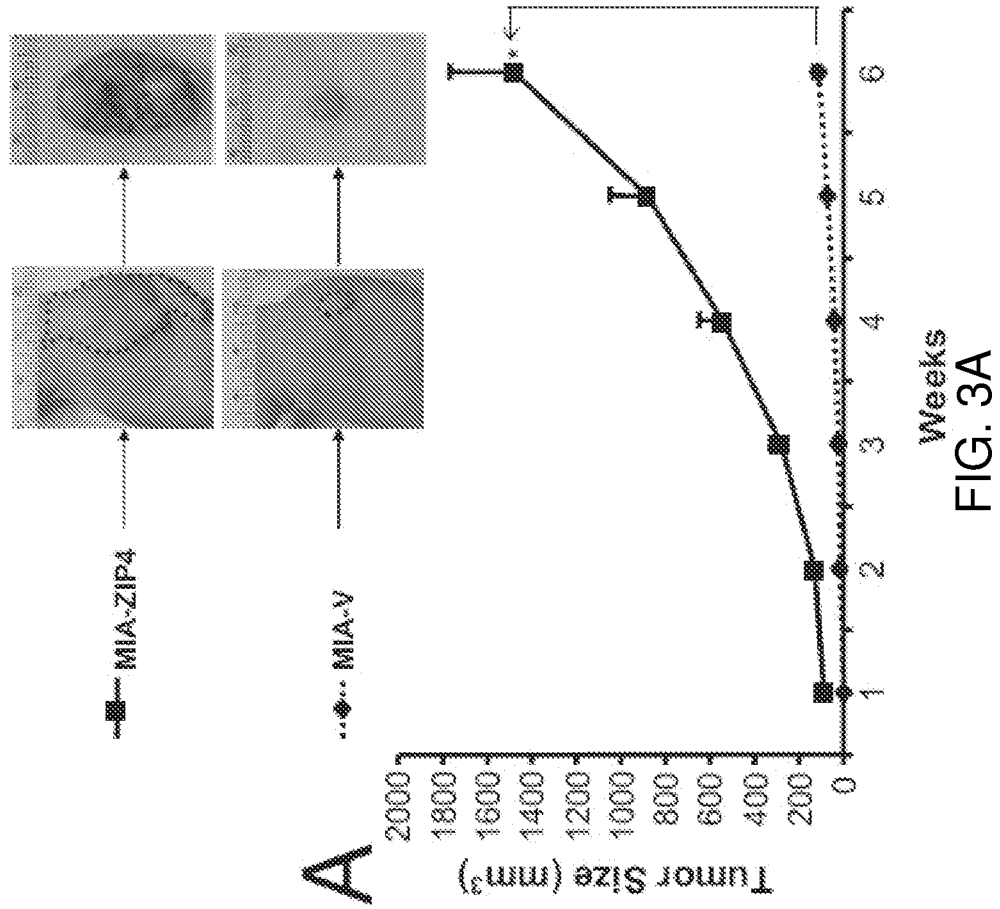
A
Tumor Size (mm³)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
1
2
3
4
5
6
Weeks
MIA-ZIP4
MIA-γ

FIG. 3A

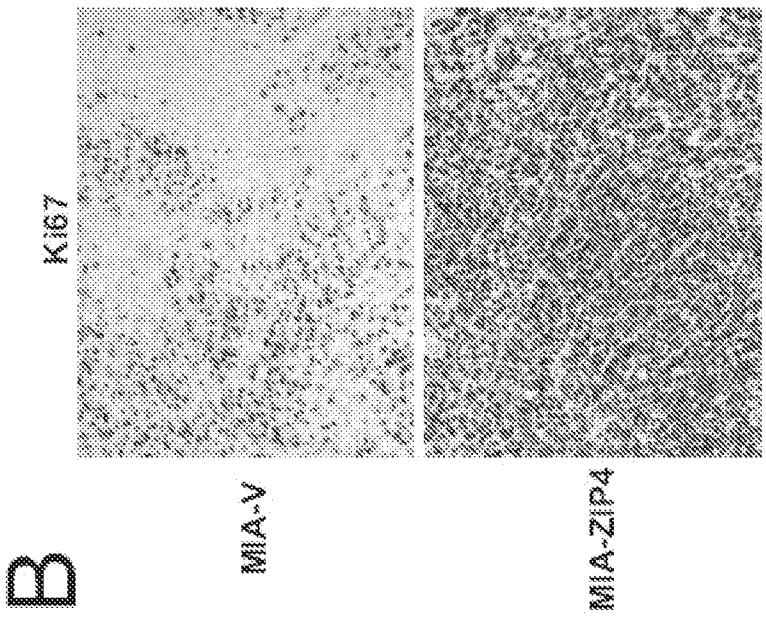

FIG. 3B

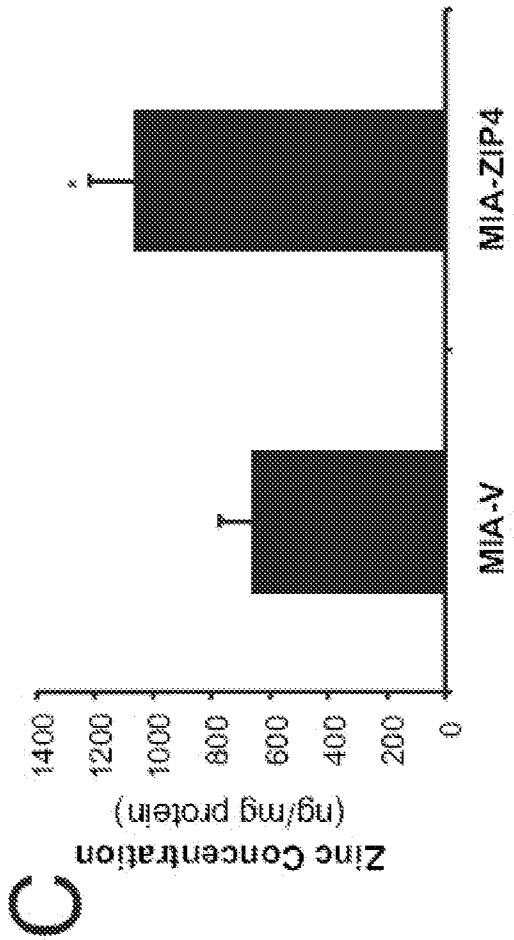
C
Zinc Concentration
(ng/mg protein)
1400
1200
1000
800
600
400
200
0
MIA-V
MIA-ZIP4
*

FIG. 3C

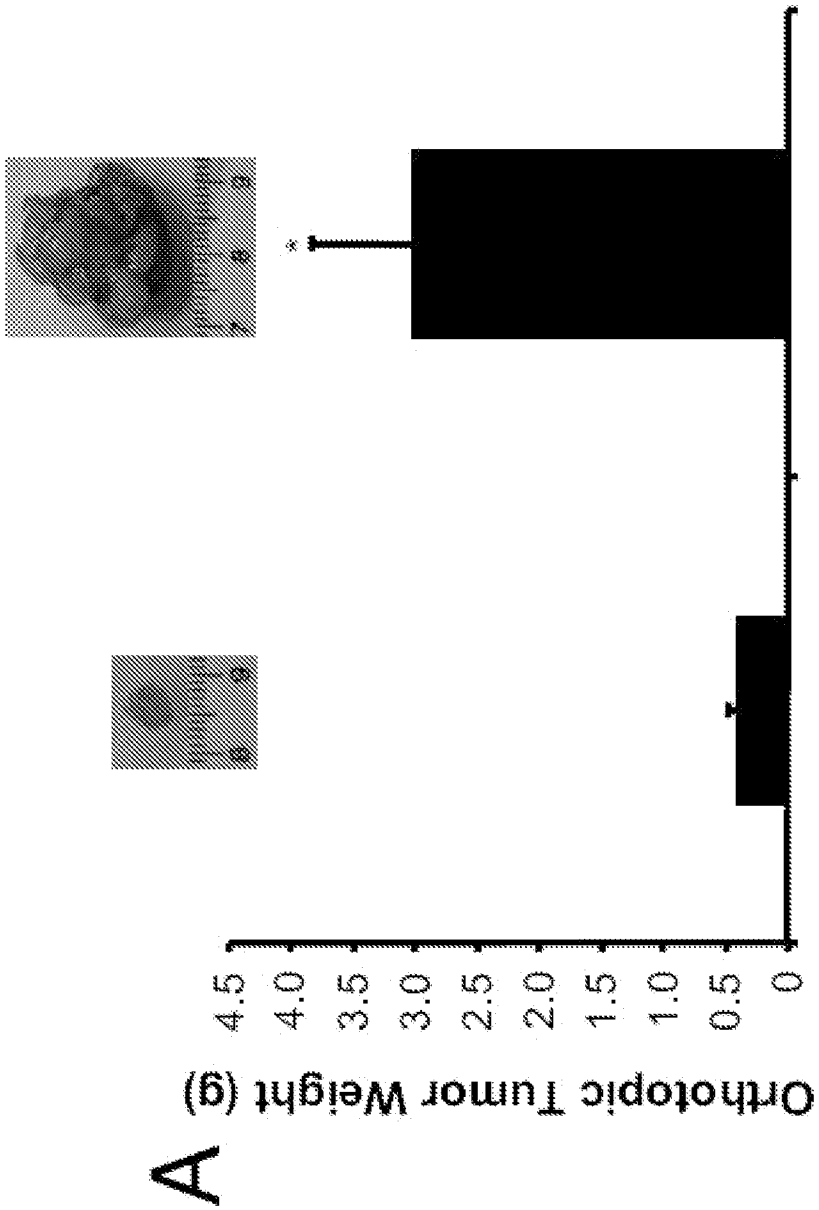
A
Orthotopic Tumor Weight (g)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
MIA-V
MIA-ZIP4

FIG. 4A

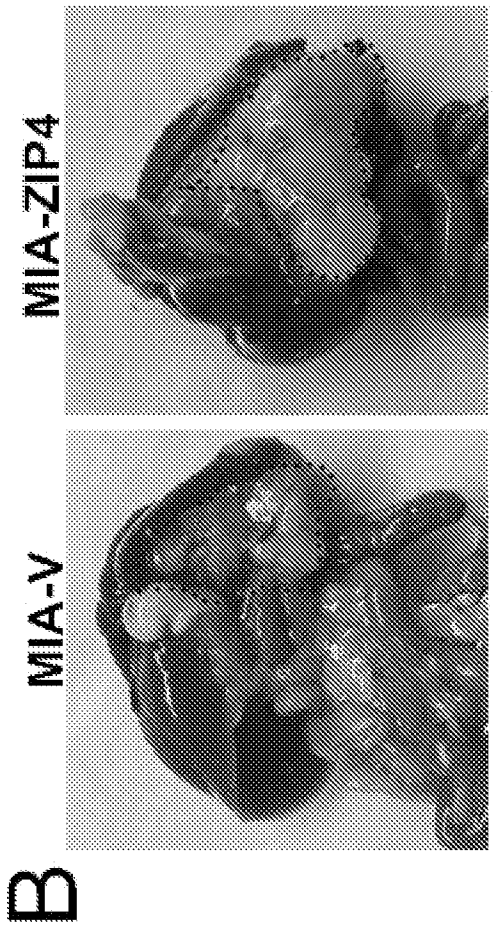

FIG. 4B

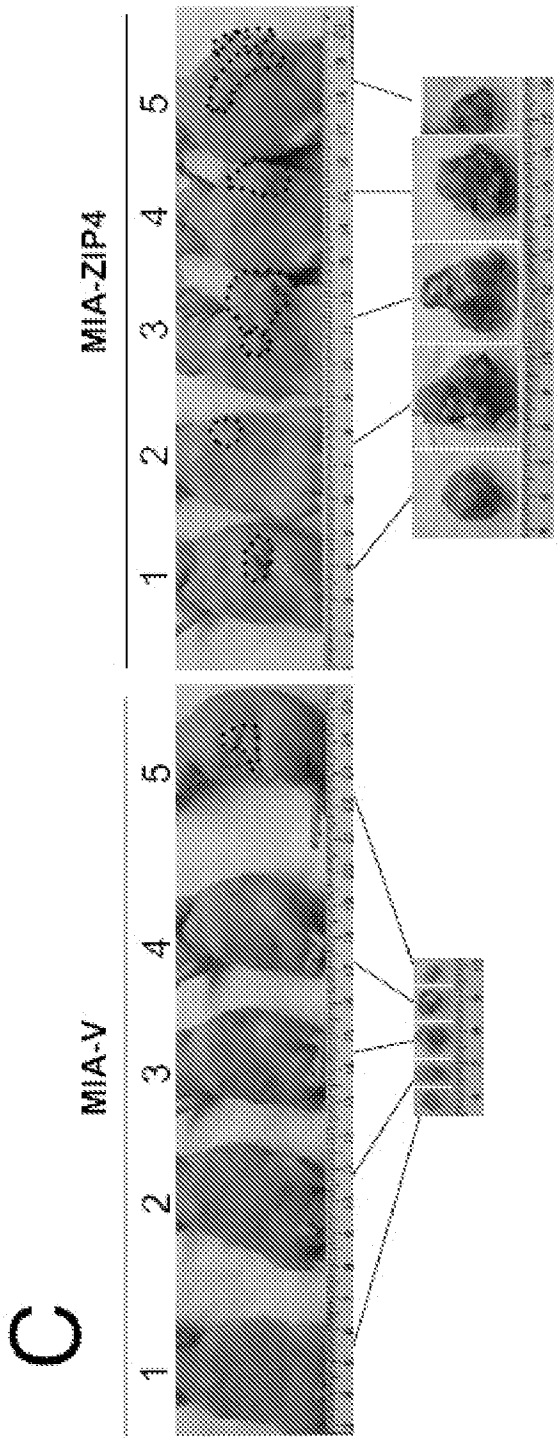

FIG. 4C

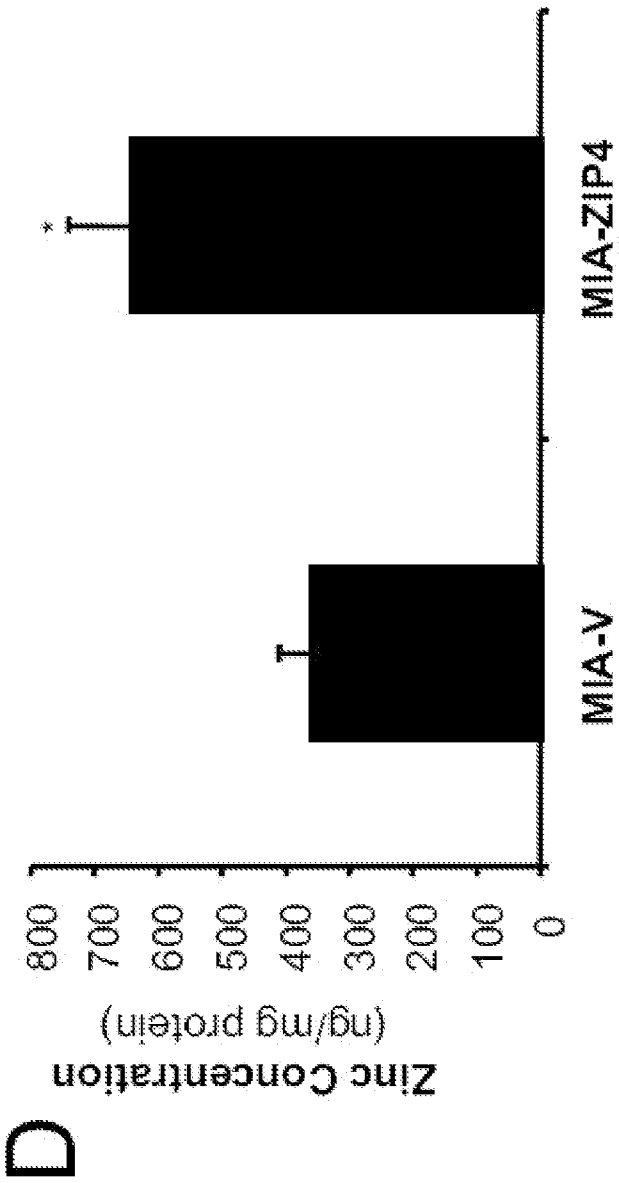
D
Zinc Concentration
(ng/mg protein)
800
700
600
500
400
300
200
100
0
MIA-V
MIA-ZIP4
*

FIG. 4D

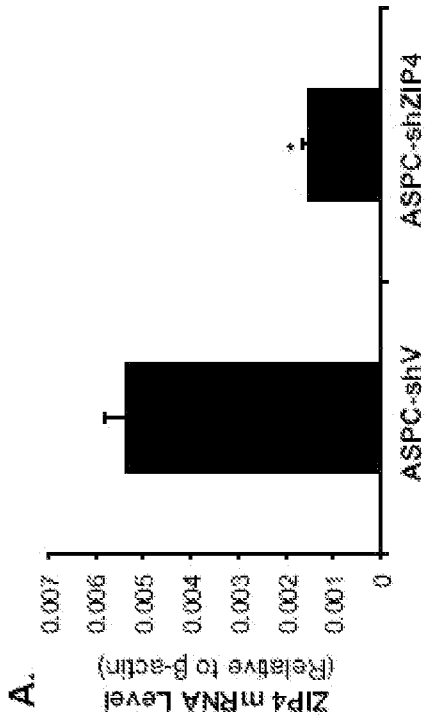
A.
ZIP4 mRNA Level
(Relative to β-actin)
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
ASPC-shV
ASPC-shZIP4

FIG. 5A

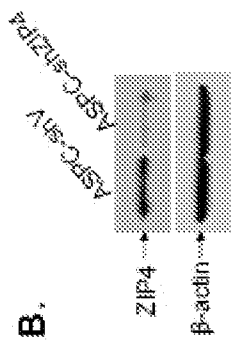

FIG. 5B

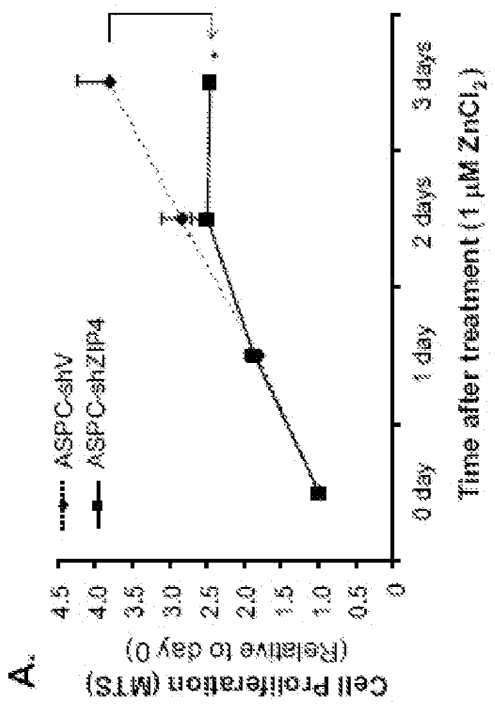
A.
Cell Proliferation (MTS)
(Relative to day 0)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
ASPC-shV
ASPC-shZIP4
0 day
1 day
2 days
3 days
Time after treatment (1 μM $ZnCl_2$)

FIG. 6A

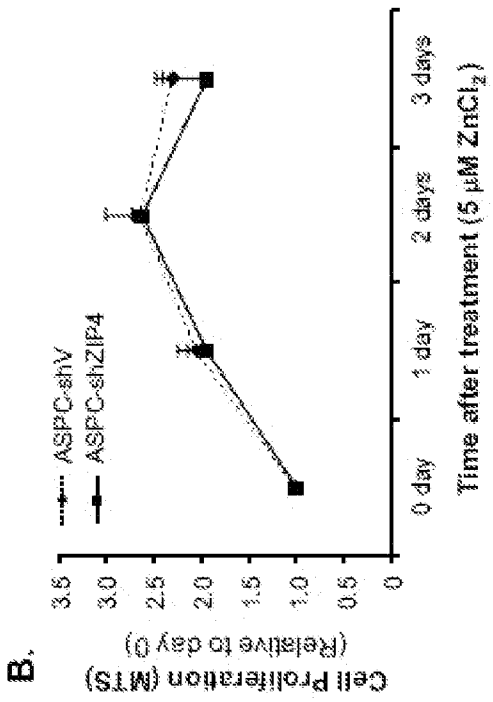
B.
Cell Proliferation (MTS)
(Relative to day 0)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
ASPC-shV
ASPC-shZIP4
0 day
1 day
2 days
3 days
Time after treatment (5 μM $ZnCl_2$)

FIG. 6B

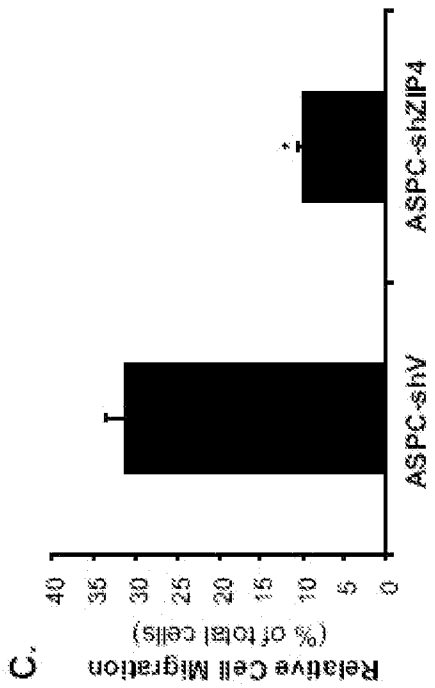
C
Relative Cell Migration
(% of total cells)
0
5
10
15
20
25
30
35
40
ASPC-shV
ASPC-shZIP4
*

FIG. 6C

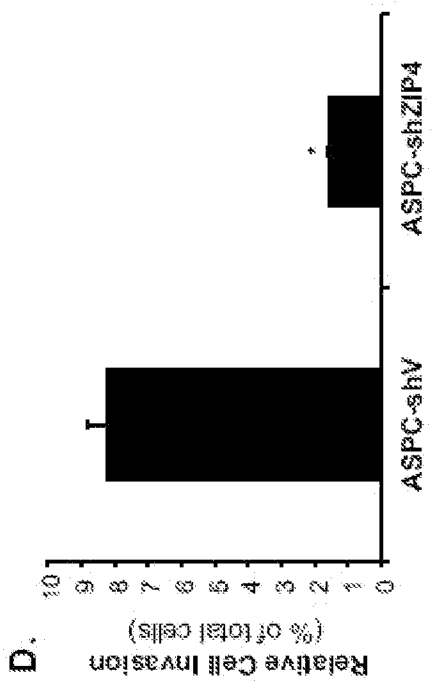
D.
Relative Cell Invasion
(% of total cells)
0
1
2
3
4
5
6
7
8
9
10
ASPC-shV
ASPC-shZIP4
*

FIG. 6D

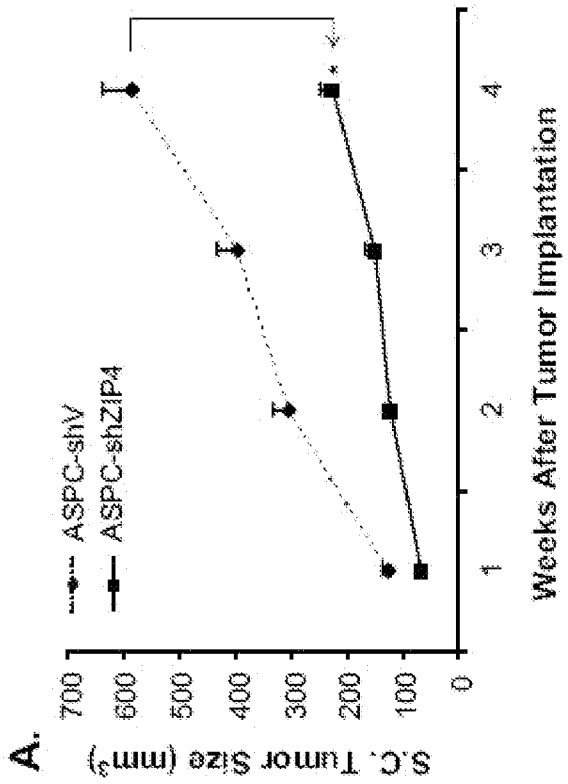
A.
S.C. Tumor Size (mm³)
700
600
500
400
300
200
100
0
1
2
3
4
Weeks After Tumor Implantation
ASPC-shV
ASPC-shZIP4

FIG. 7A

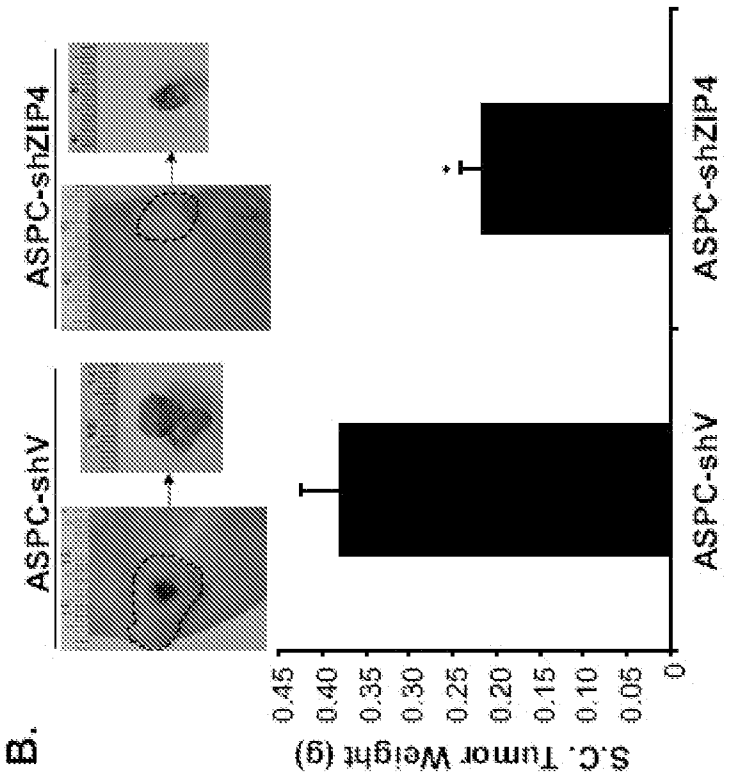
B.
ASPC-shV
ASPC-shZIP4
S.C. Tumor Weight (g)
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
ASPC-shV
ASPC-shZIP4
*

FIG. 7B

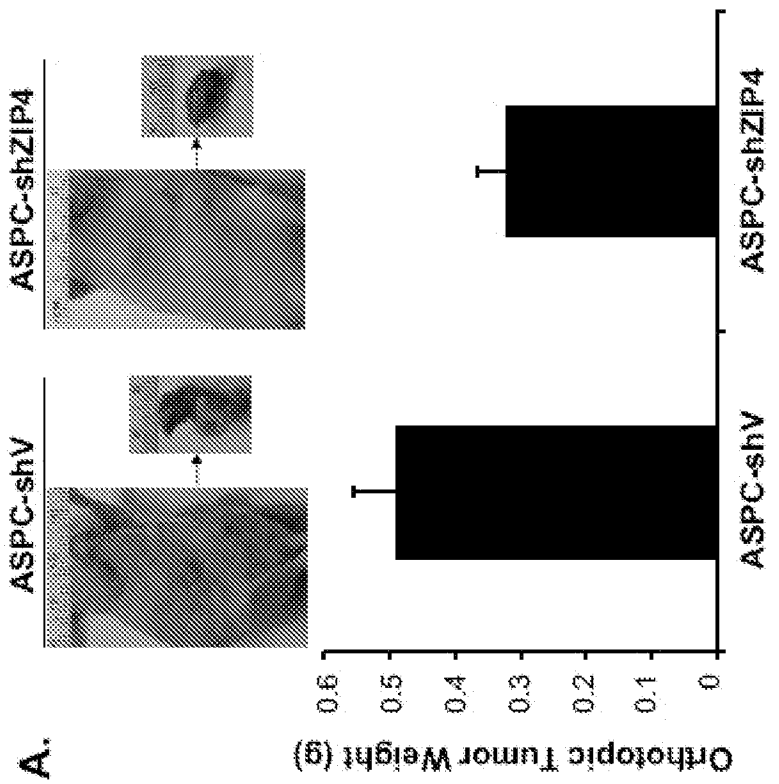
A.
ASPC-shV
ASPC-shZIP4
Orthotopic Tumor Weight (g)
0.6
0.5
0.4
0.3
0.2
0.1
0
ASPC-shV
ASPC-shZIP4

FIG. 8A

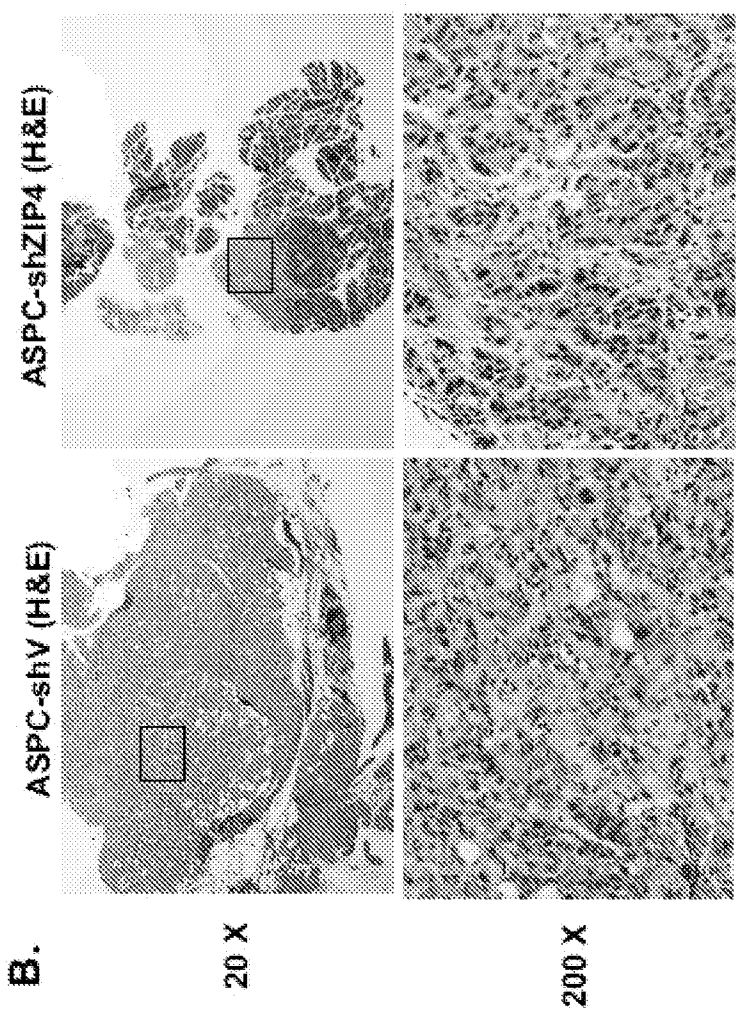

FIG. 8B

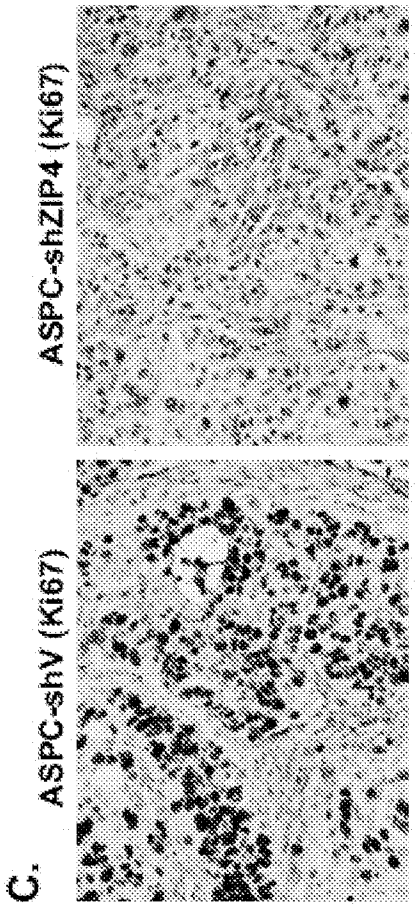

FIG. 8C

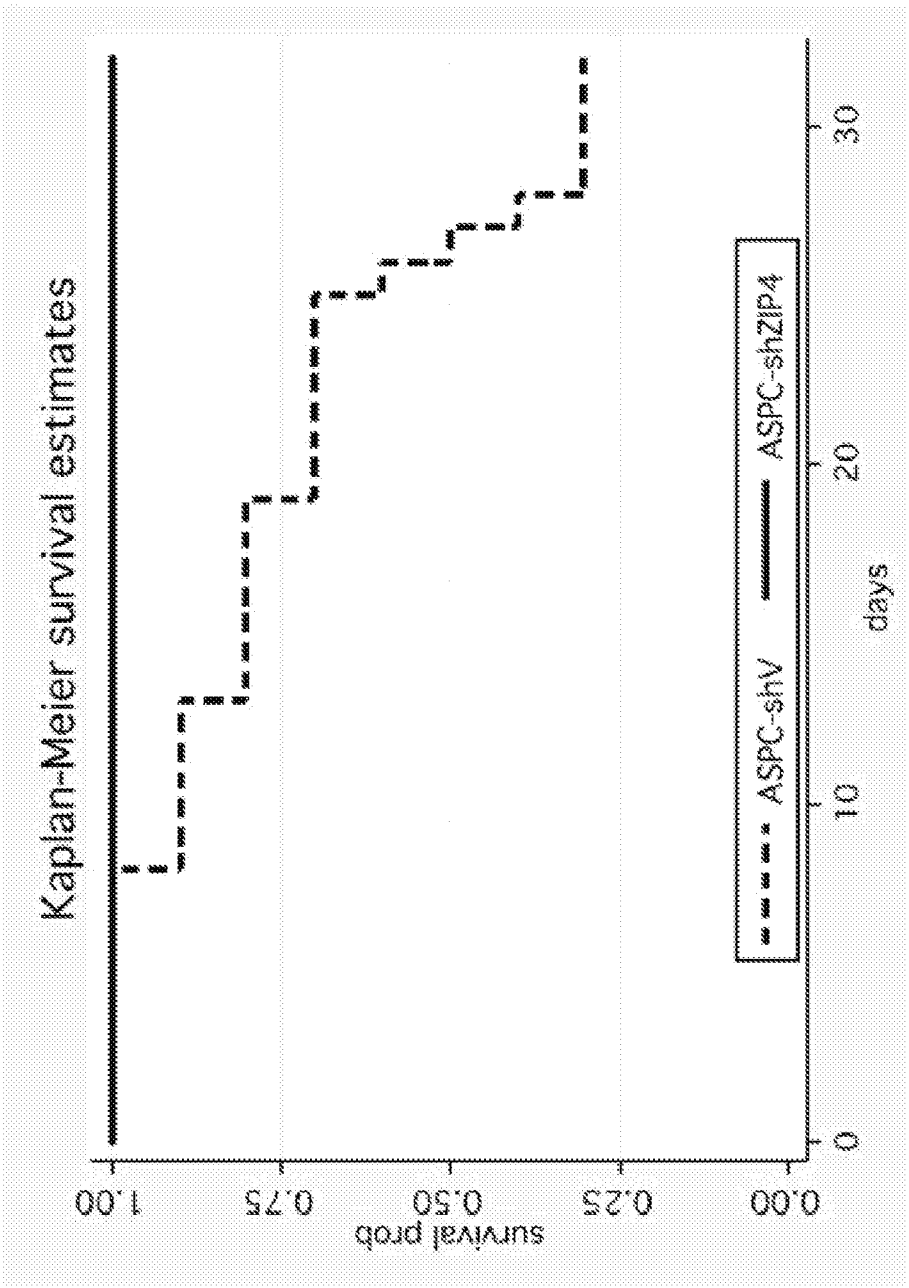
Kaplan-Meier survival estimates
survival prob
0.00
0.25
0.50
0.75
1.00
0
10
20
30
days
ASPC-shV
ASPC-shZIP4

FIG. 8D

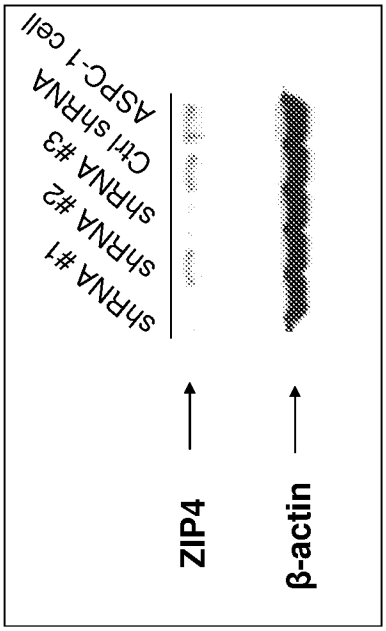

FIG. 9A

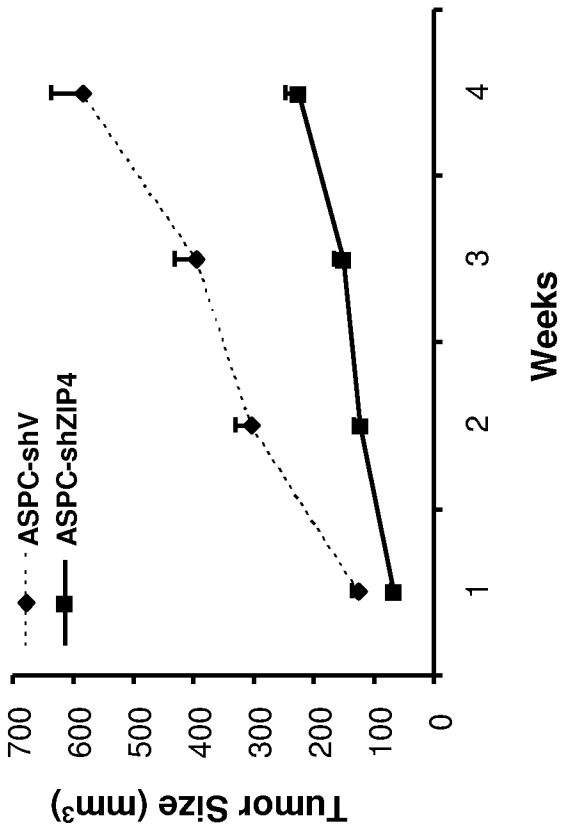
Tumor Size (mm³)
700
600
500
400
300
200
100
0
1
2
3
4
Weeks
ASPC-shV
ASPC-shZIP4

FIG. 9B

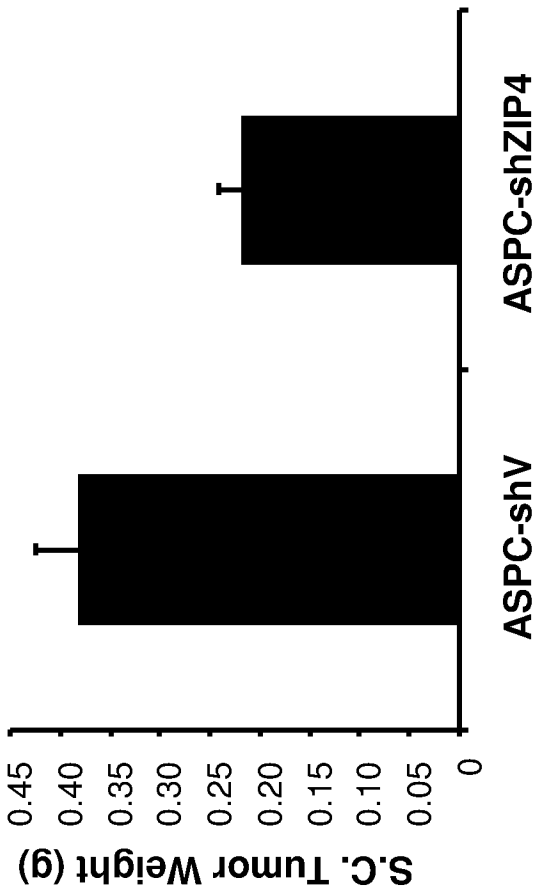
S.C. Tumor Weight (g)
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
ASPC-shV
ASPC-shZIP4

FIG. 9C

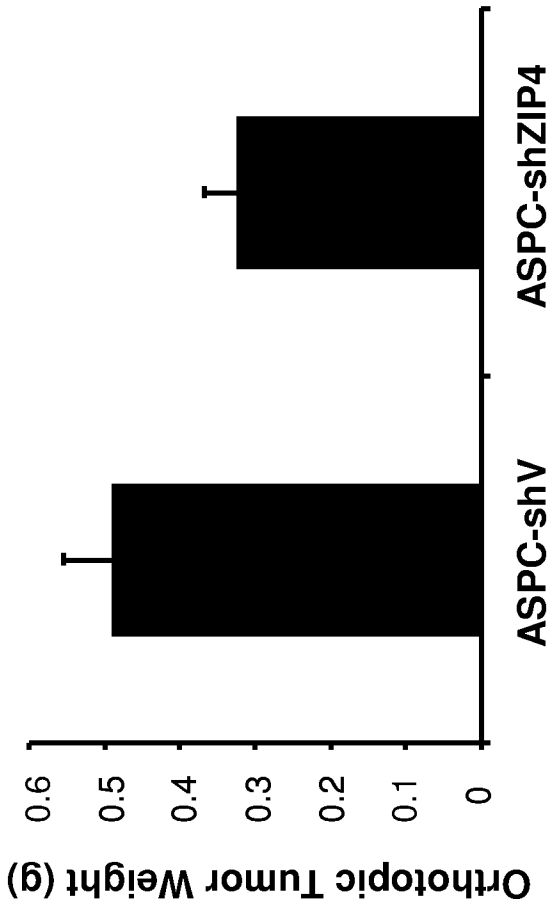
Orthotopic Tumor Weight (g)
0.6
0.5
0.4
0.3
0.2
0.1
0
ASPC-shV
ASPC-shZIP4

FIG. 9D

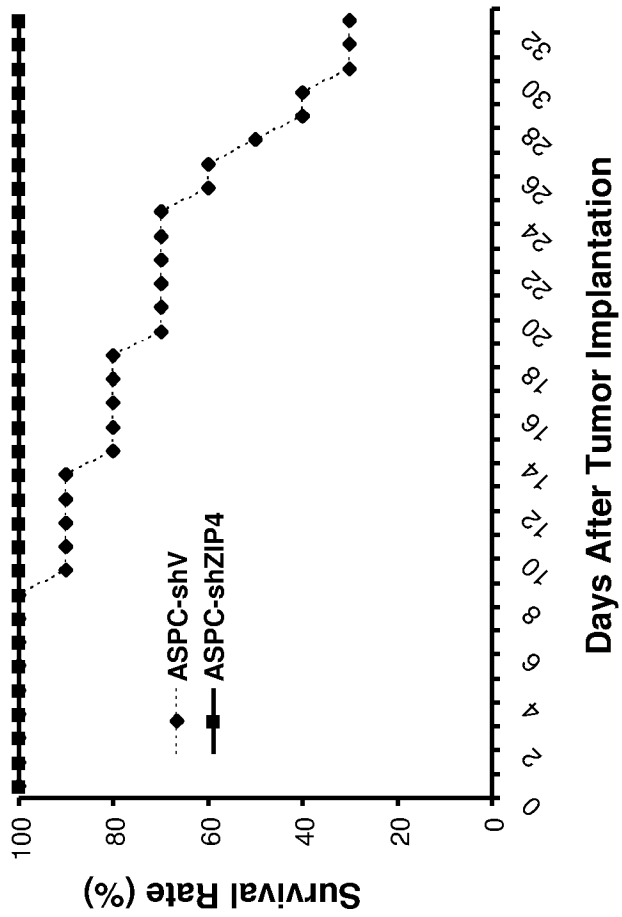
Survival Rate (%)
100
80
60
40
20
0
ASPC-shV
ASPC-shZIP4
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
Days After Tumor Implantation

FIG. 9E

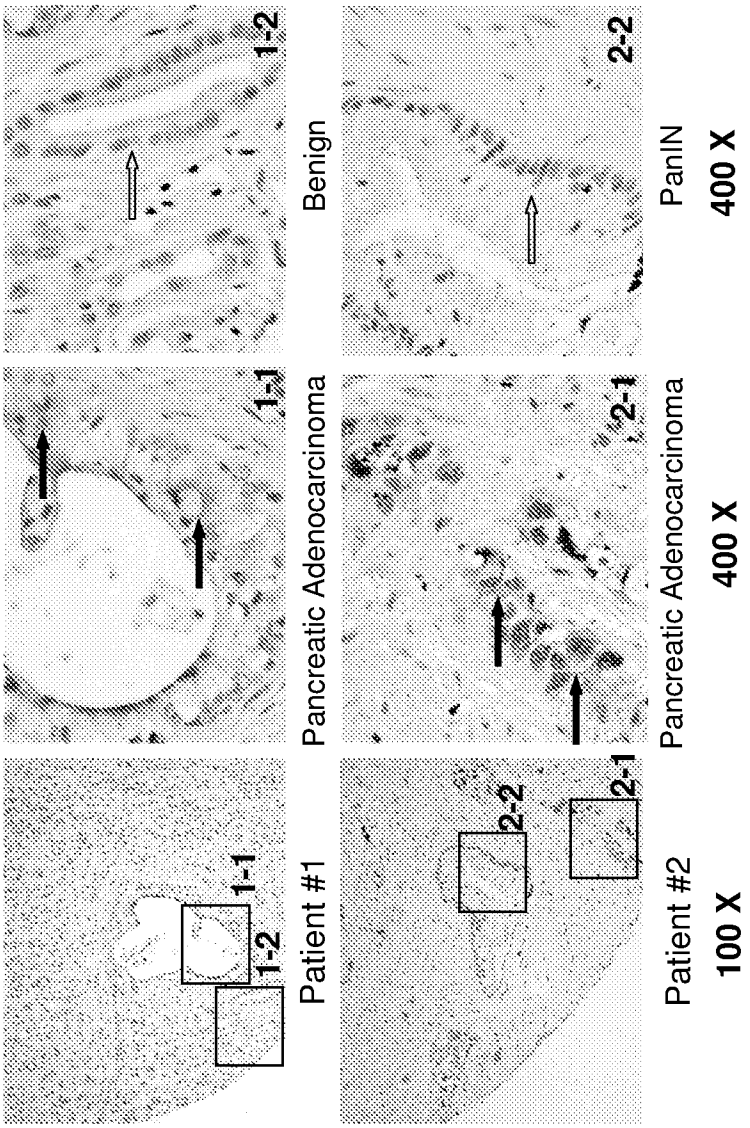
1-2
1-1
1-2
Benign
Pancreatic Adenocarcinoma
Patient #1
100 X
2-2
2-1
2-2
2-1
PanIN
Pancreatic Adenocarcinoma
Patient #2
400 X
400 X

FIG. 10

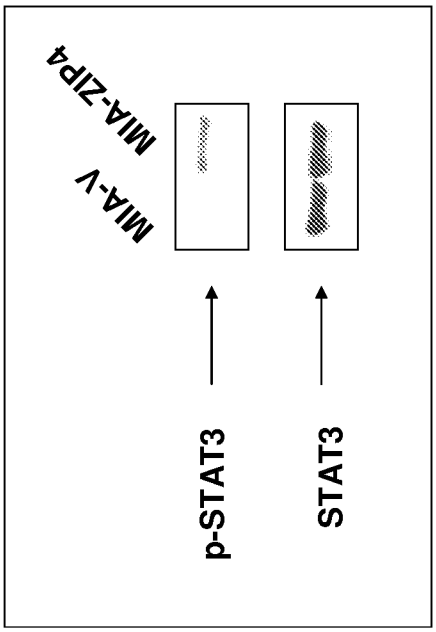

FIG. 11

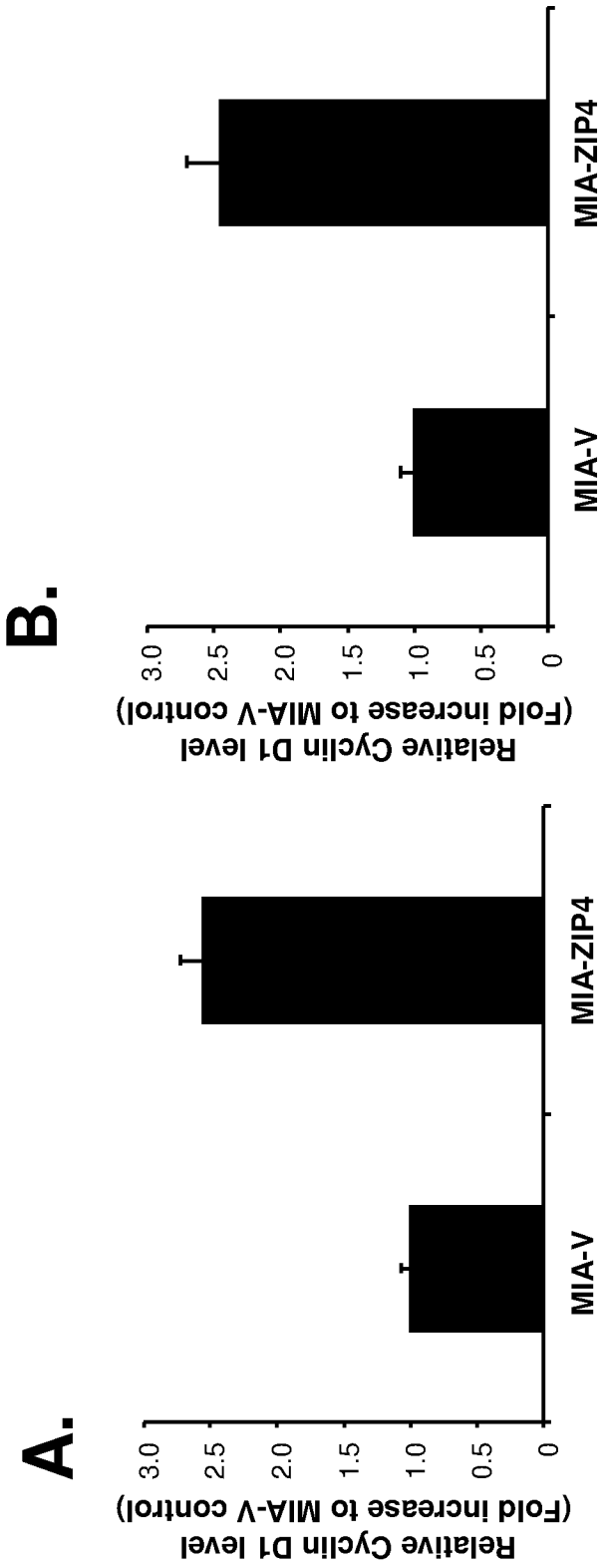
A.
Relative Cyclin D1 level
(Fold increase to MIA-V control)
3.0
2.5
2.0
1.5
1.0
0.5
0
MIA-V
MIA-ZIP4
B.
Relative Cyclin D1 level
(Fold increase to MIA-V control)
3.0
2.5
2.0
1.5
1.0
0.5
0
MIA-V
MIA-ZIP4

FIG. 12

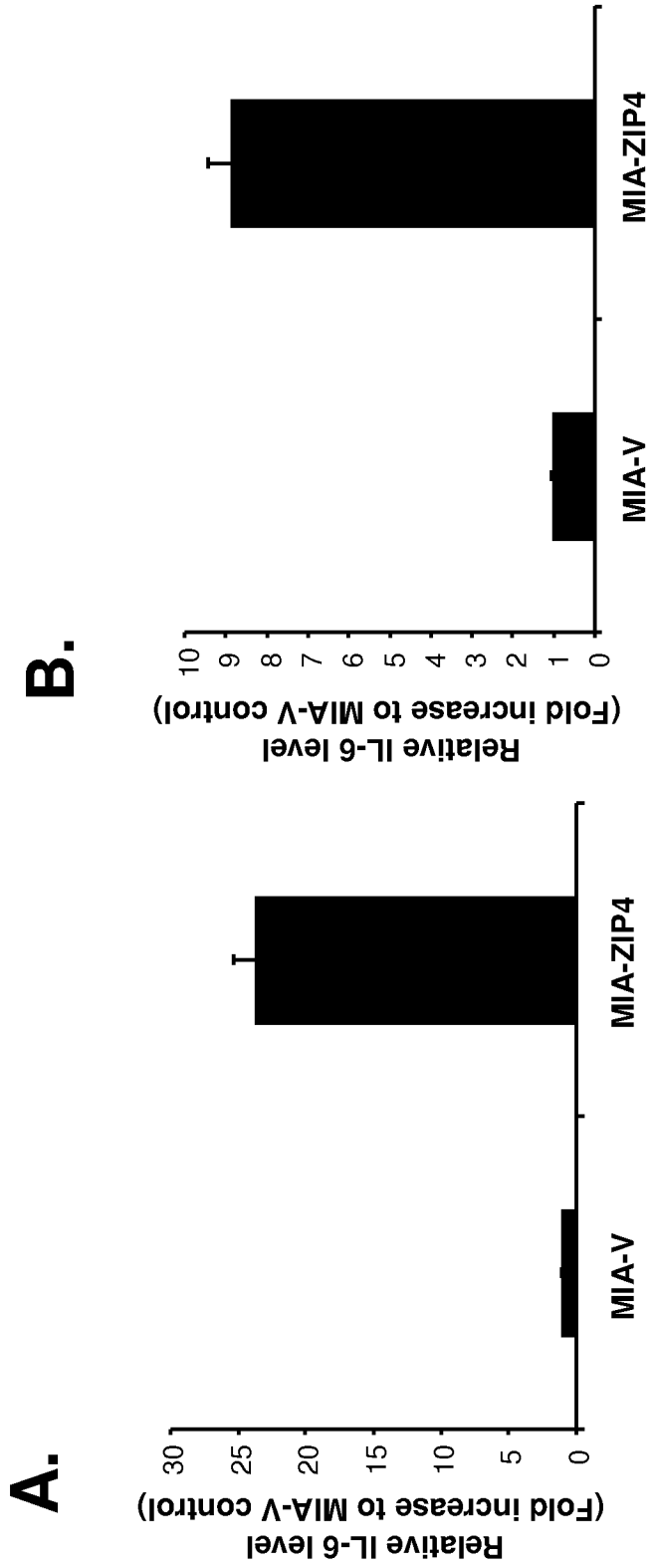
A.
Relative IL-6 level
(Fold increase to MIA-V control)
0
5
10
15
20
25
30
MIA-V
MIA-ZIP4
B.
Relative IL-6 level
(Fold increase to MIA-V control)
0
1
2
3
4
5
6
7
8
9
10
MIA-V
MIA-ZIP4

FIG. 13

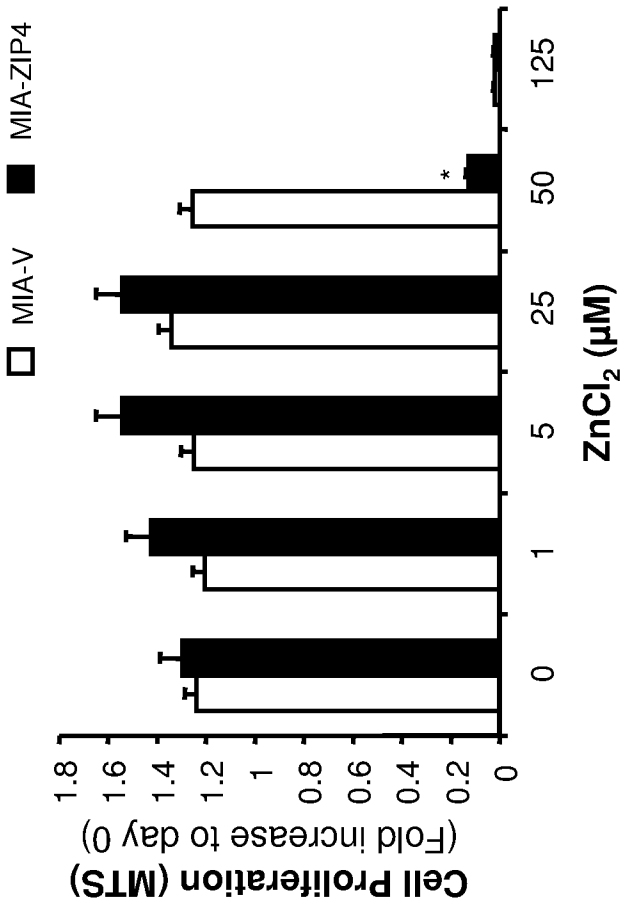
MIA-V
MIA-ZIP4
Cell Proliferation (MTS)
(Fold increase to day 0)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
1
5
25
50
125
$ZnCl_2$ (μM)
*

FIG. 14

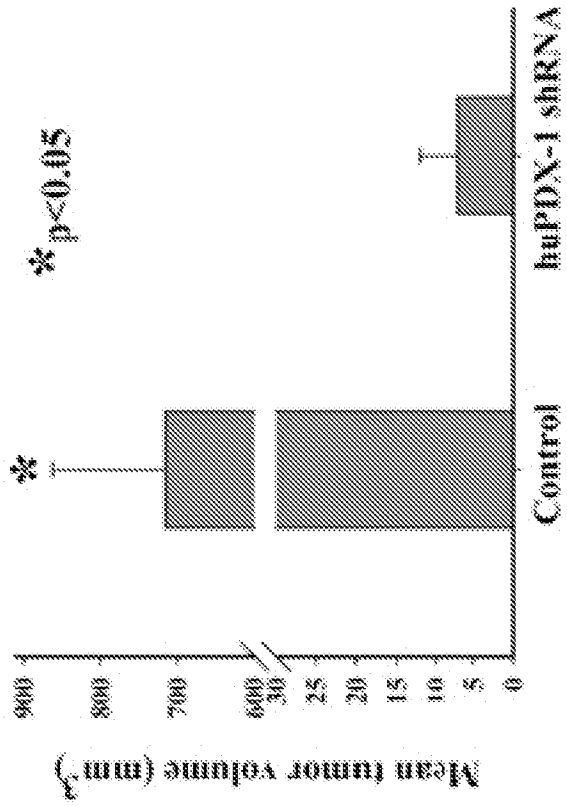
Mean tumor volume (mm³)
900
800
700
600
30
25
20
15
10
5
0
*
*p<0.05
Control
huPDX-1 shRNA
Treatments

FIG. 15

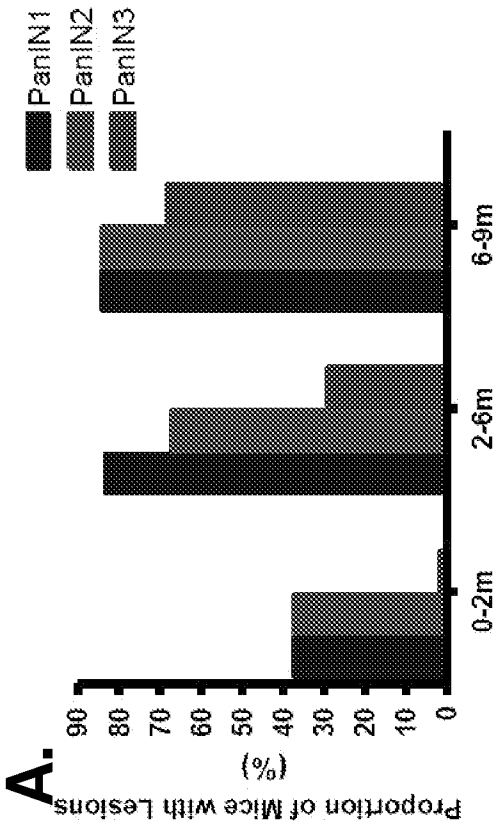
A.
Proportion of Mice with Lesions
(%)
0
10
20
30
40
50
60
70
80
90
0-2m
2-6m
6-9m
PanIN1
PanIN2
PanIN3

FIG. 16A

B.

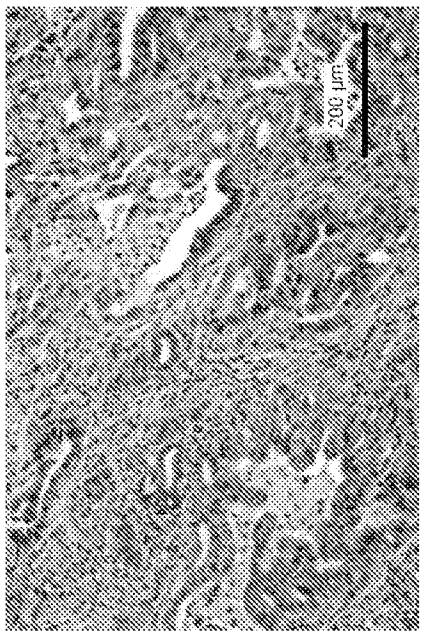

FIG. 16B

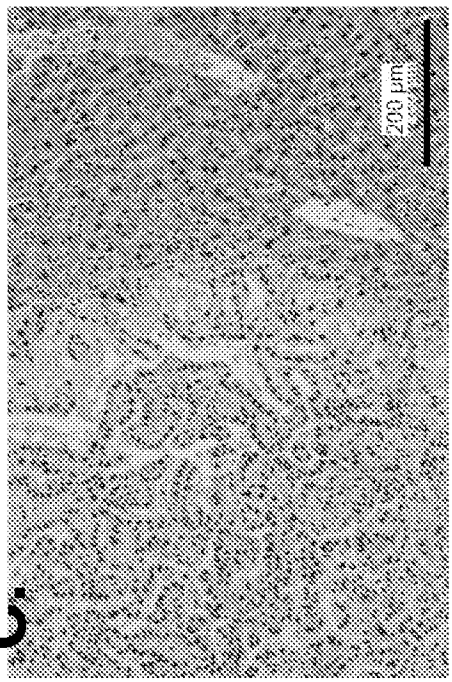

FIG. 16C

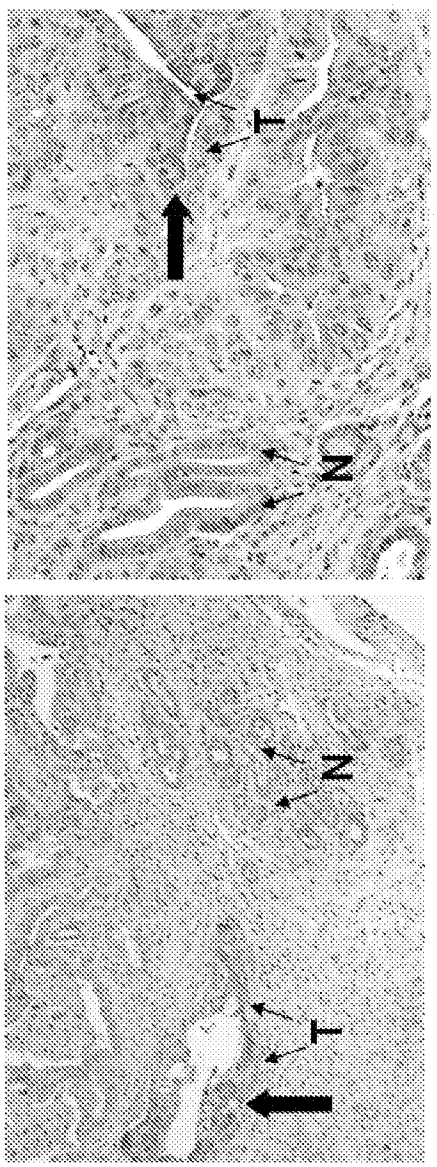

FIG. 17

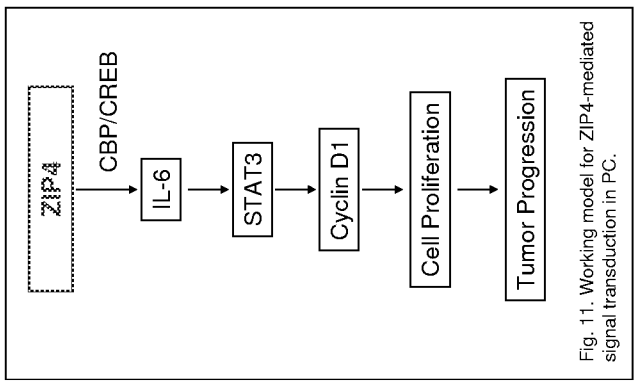

Fig. 11. Working model for ZIP4-mediated signal transduction in PC.

FIG18