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(54) **PROTEIN KINASE C IOTA**

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8, 2004.

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A61K 31/352	(2006.01)
A61P 35/00	(2006.01)

(52) **U.S. Cl.** **424/649**; 514/359; 514/282; 514/496;
514/256; 514/576; 514/453

(57) **ABSTRACT**

The invention involves PKC_ι signaling. The invention pro-
vides, for example, transgenic animals, inhibitors of PKC_ι
signaling, methods for inhibiting PKC_ι signaling, methods
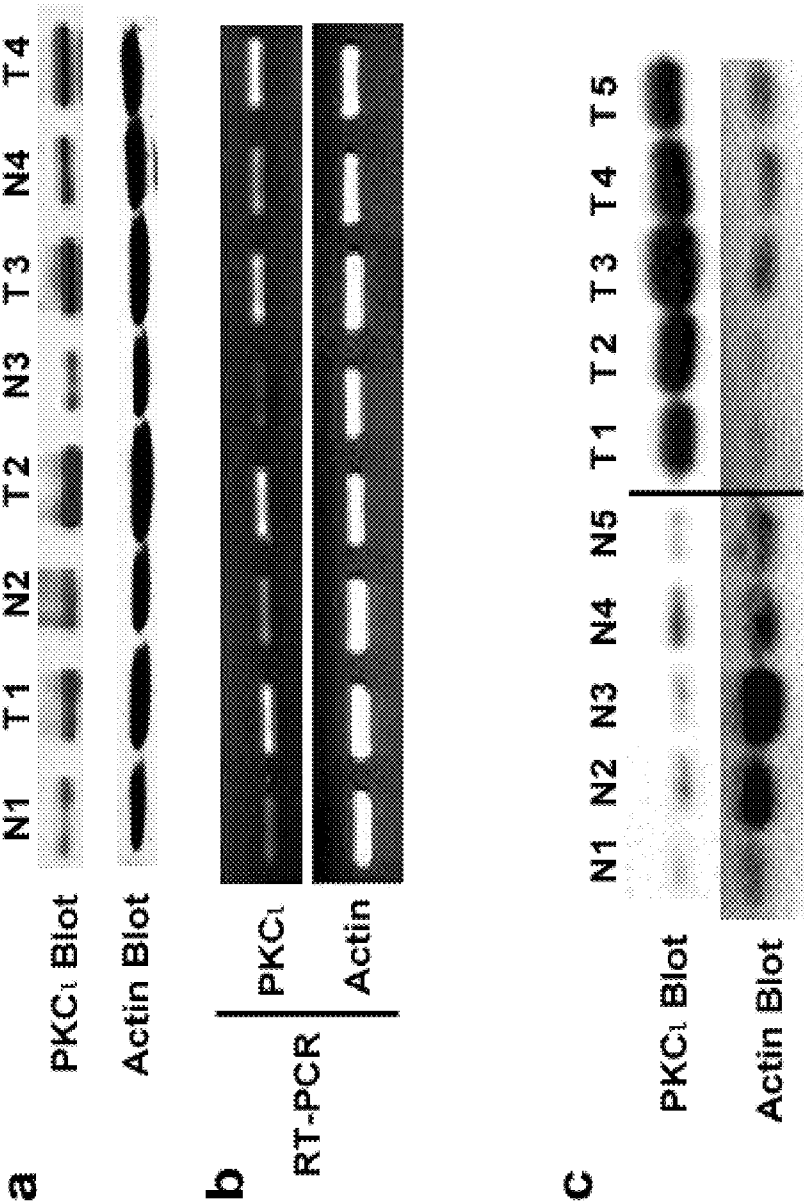for identifying inhibitors of PKC_ι signaling, and methods for
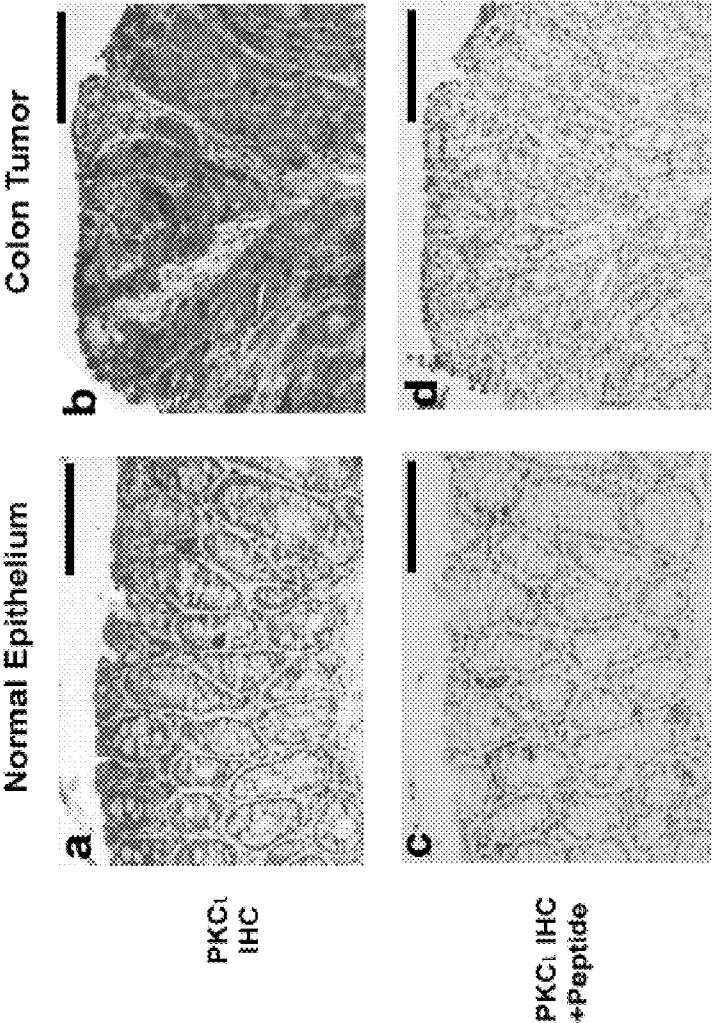diagnosing cancer.

Figure 1
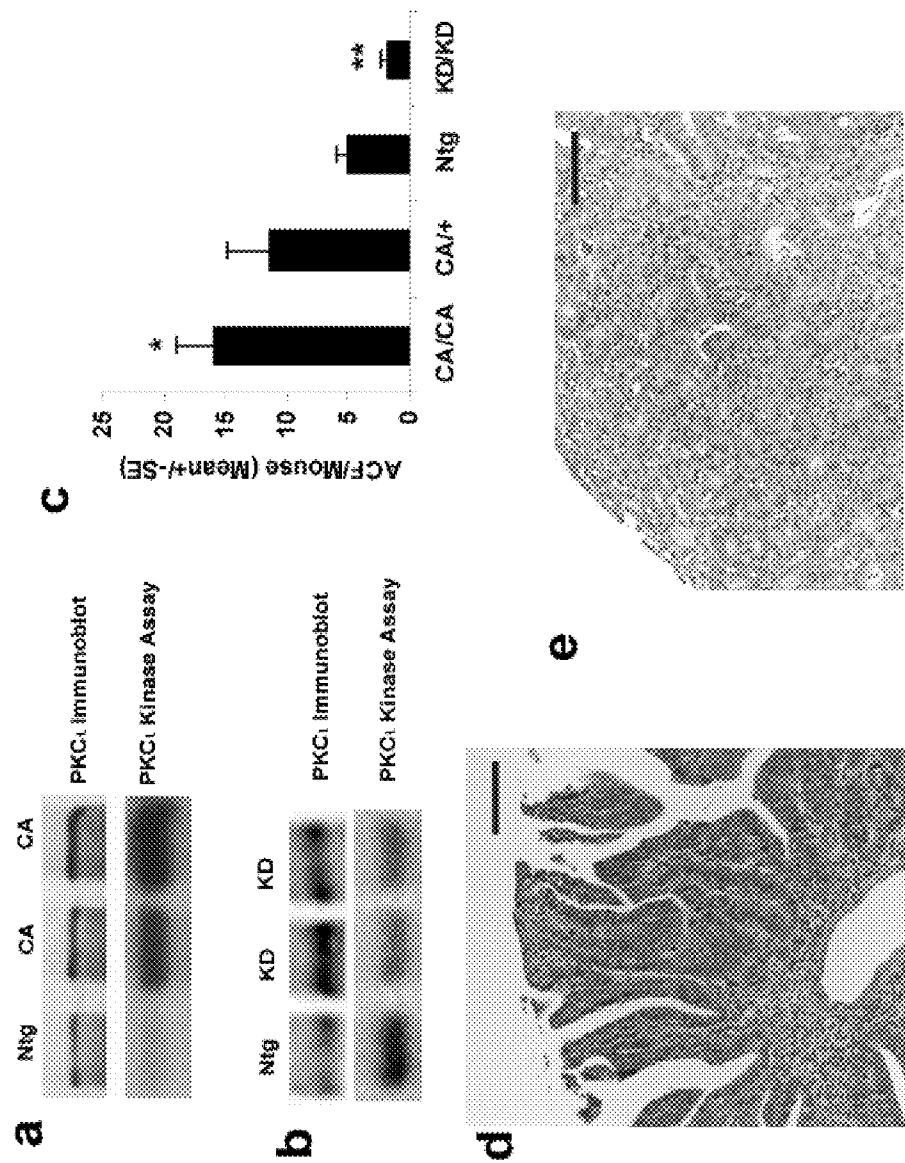
Figure 2

Figure 3

Figure 4
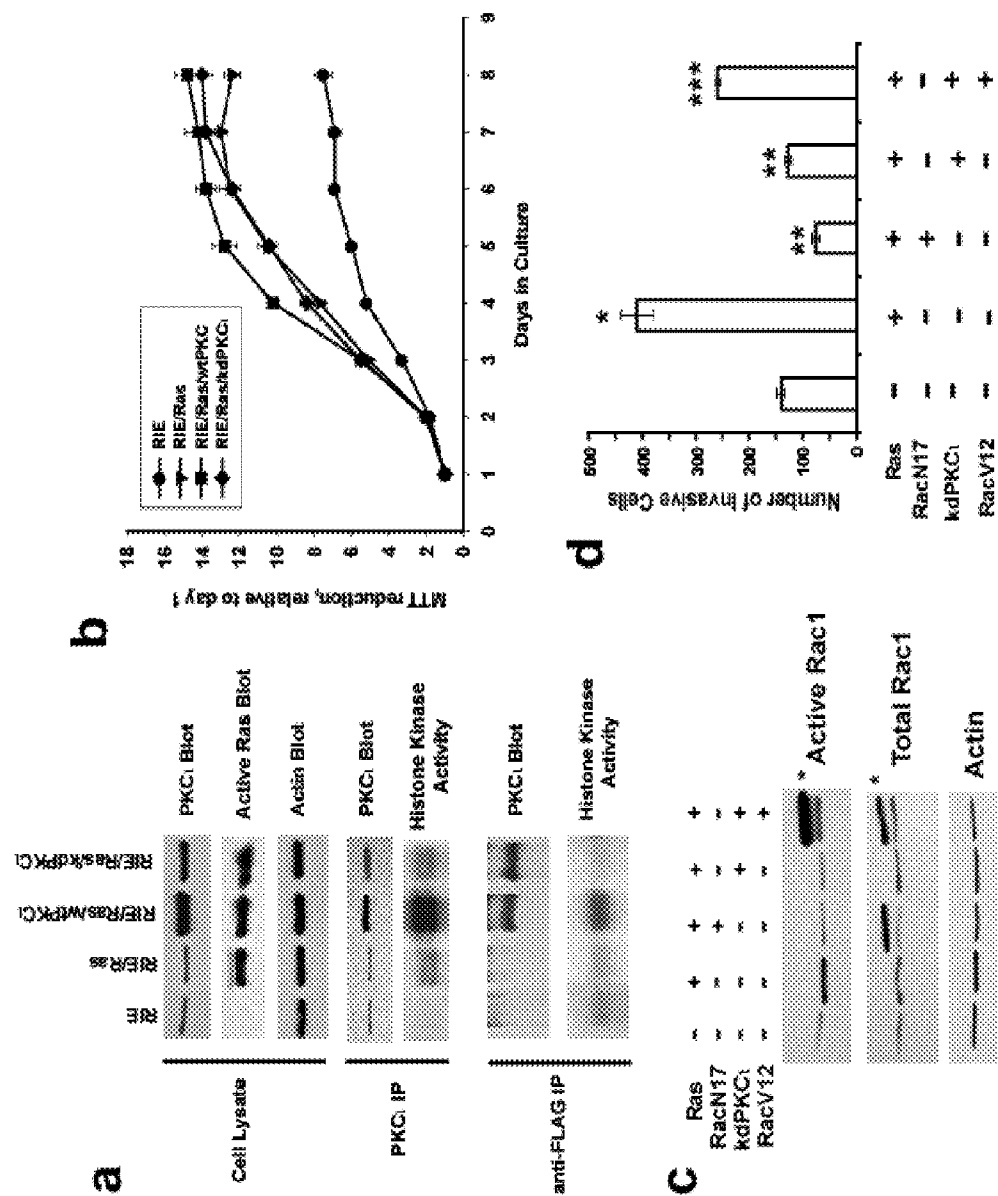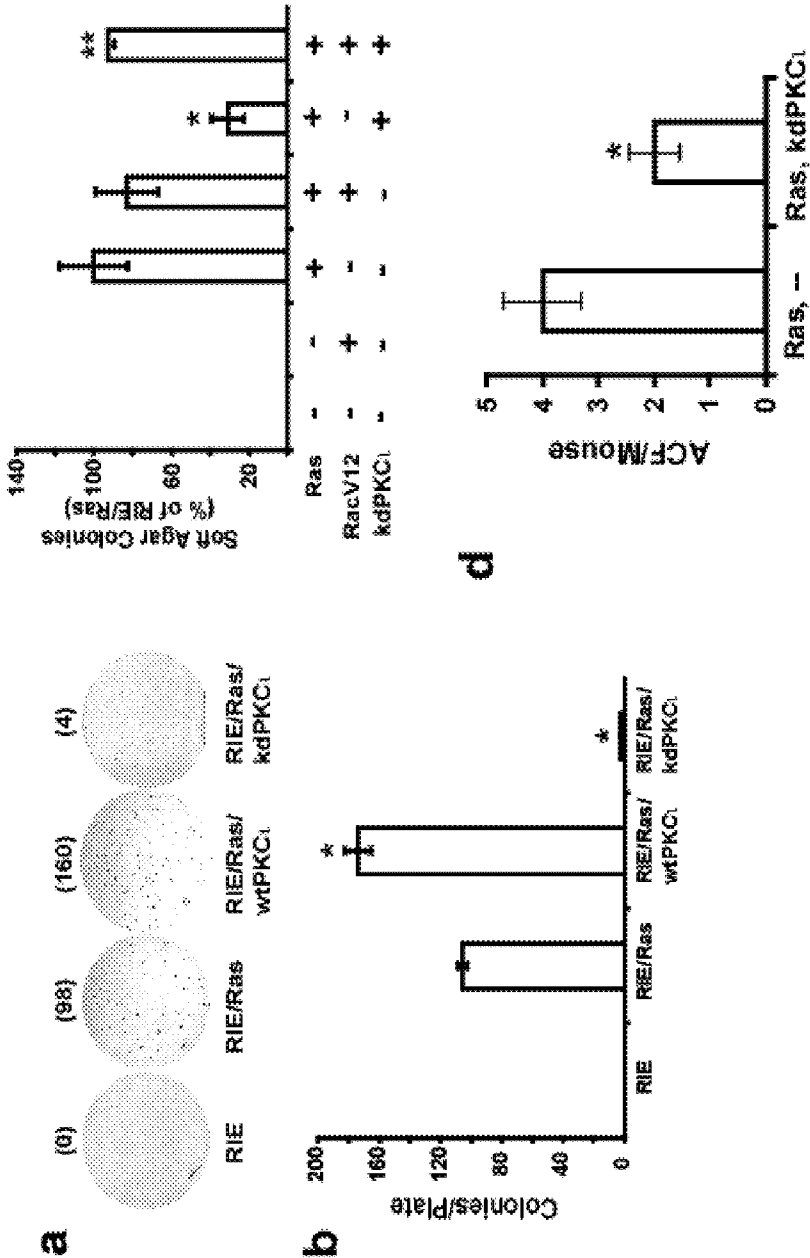
Figure 5

Figure 6

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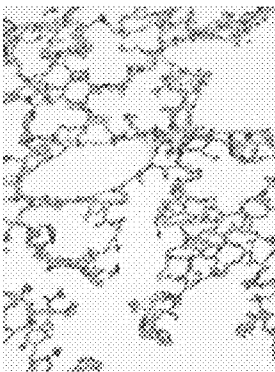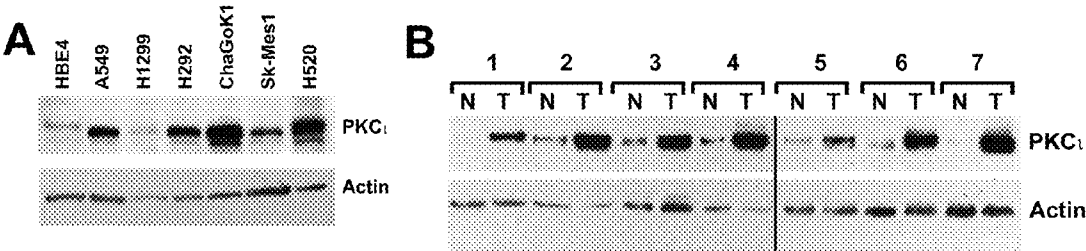# Figure 7

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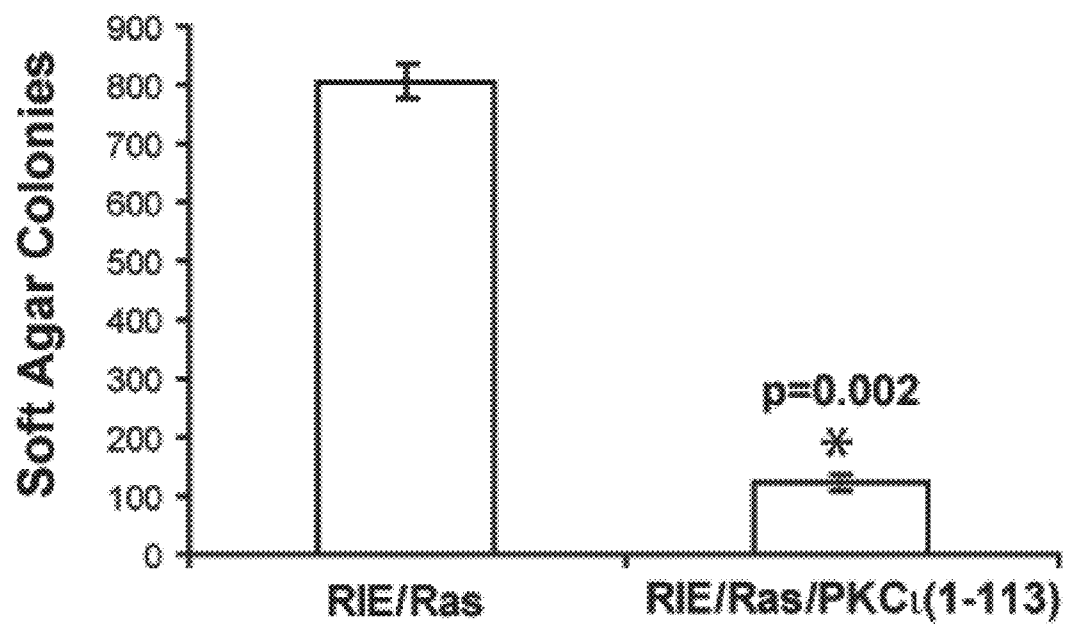
Figure 8

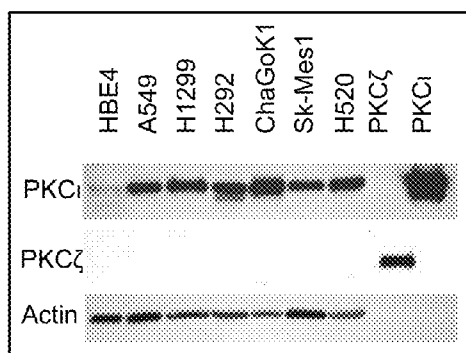**Normal Lung Epithelium**
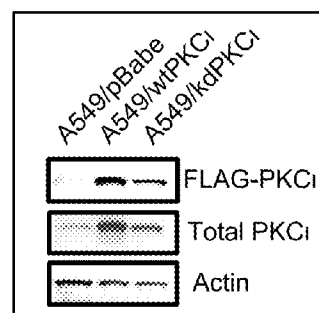
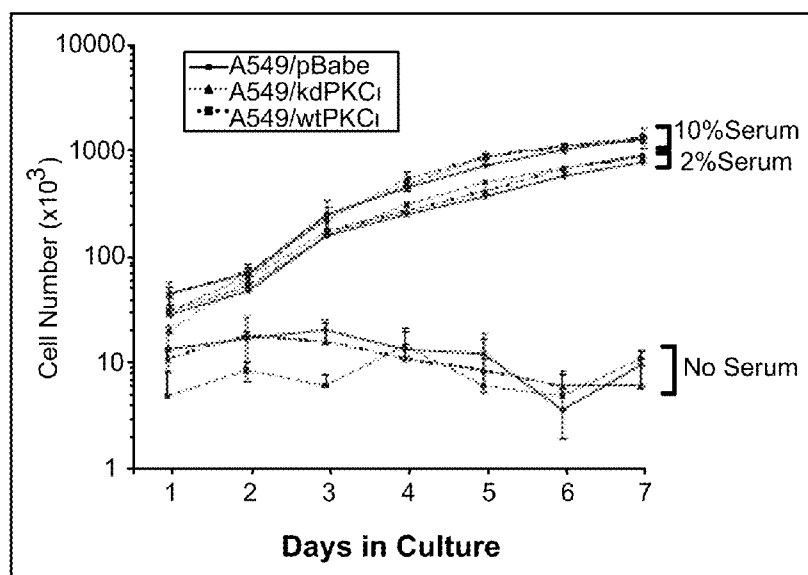**Adenocarcinoma**

Squamous Cell Carcinoma
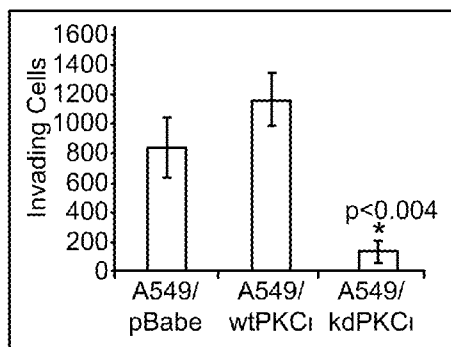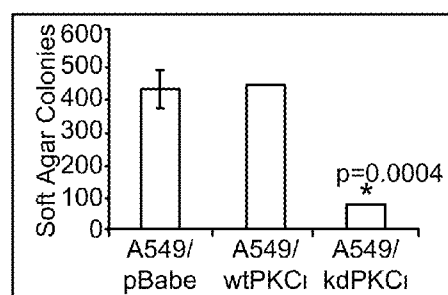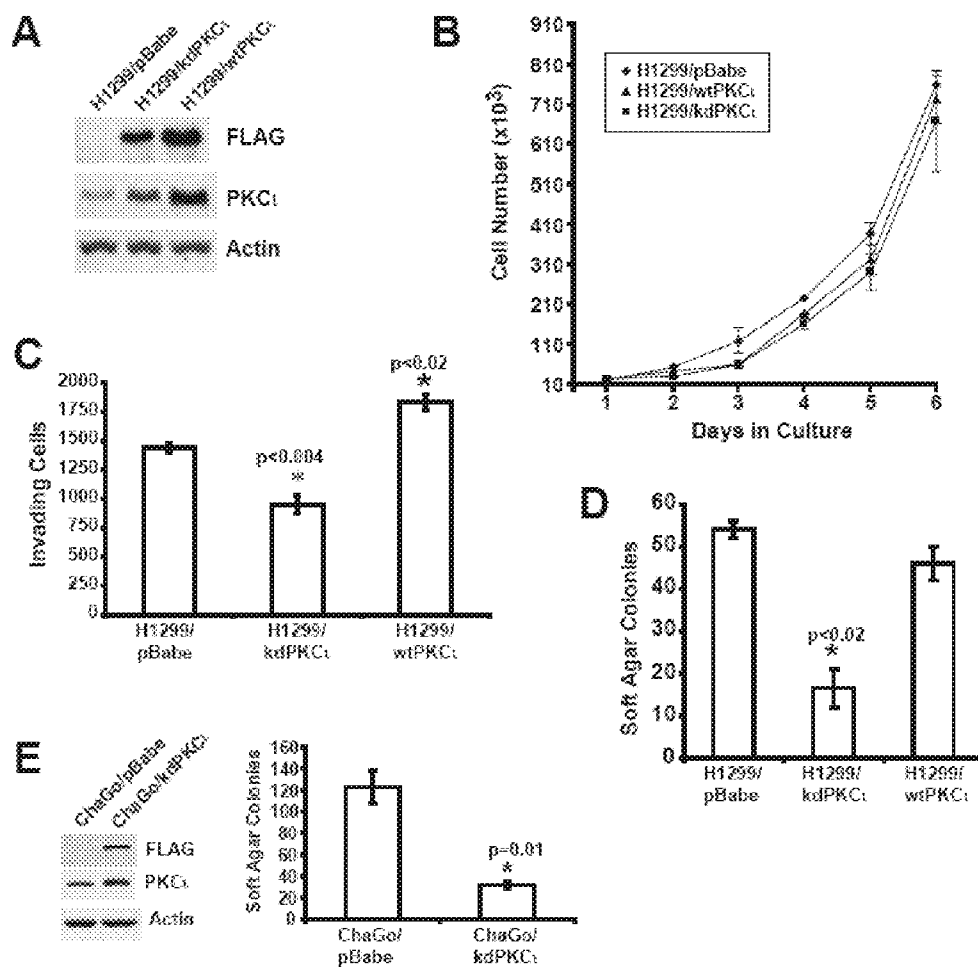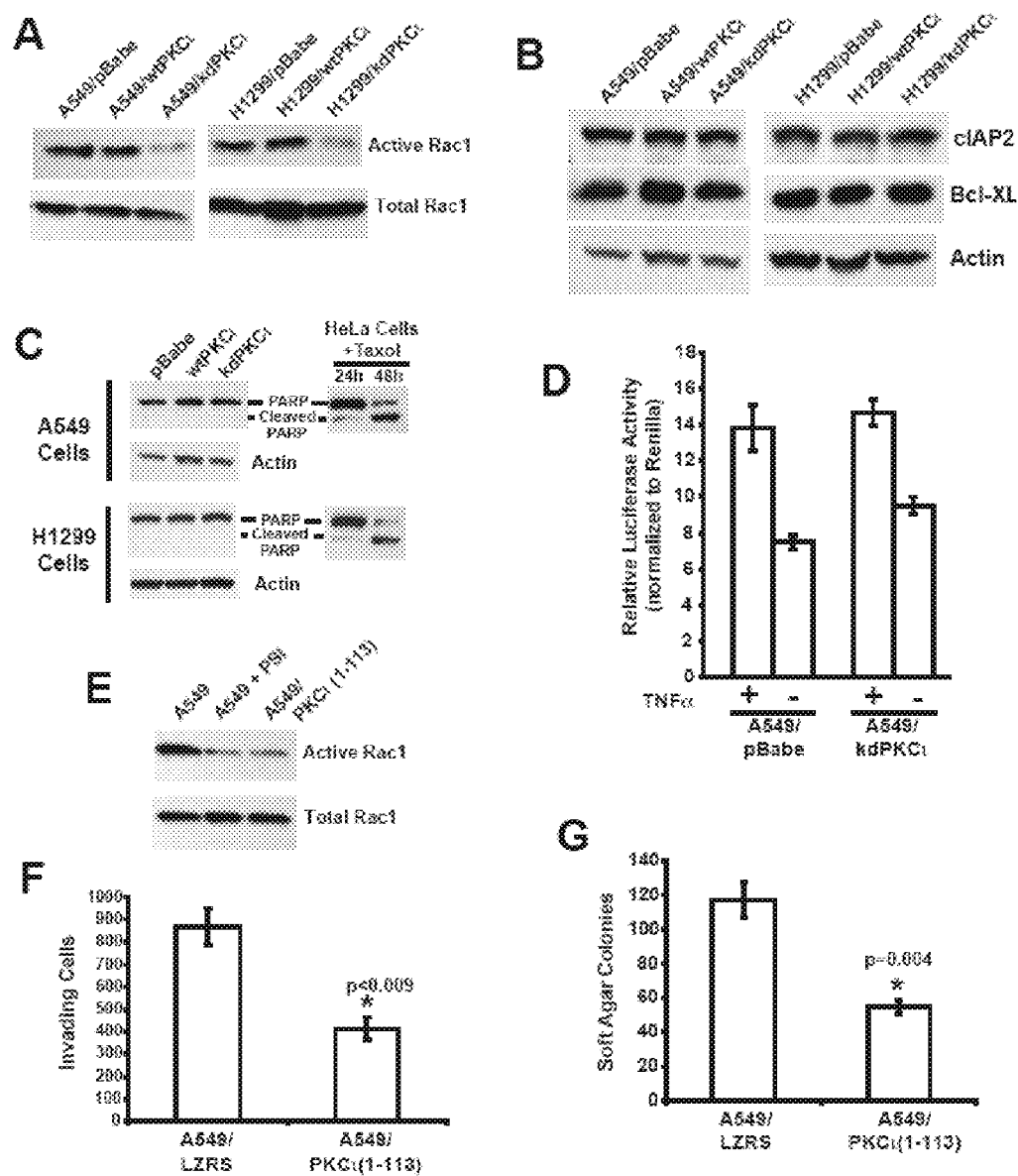
Figure 9

FIG. 10A
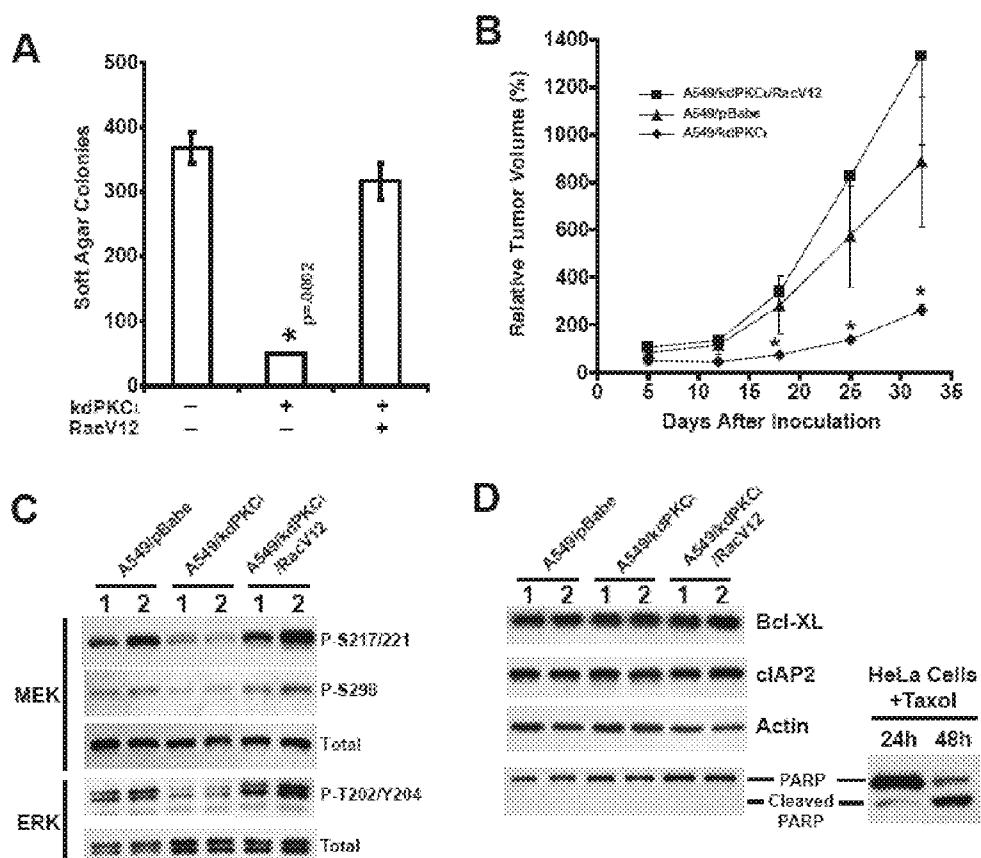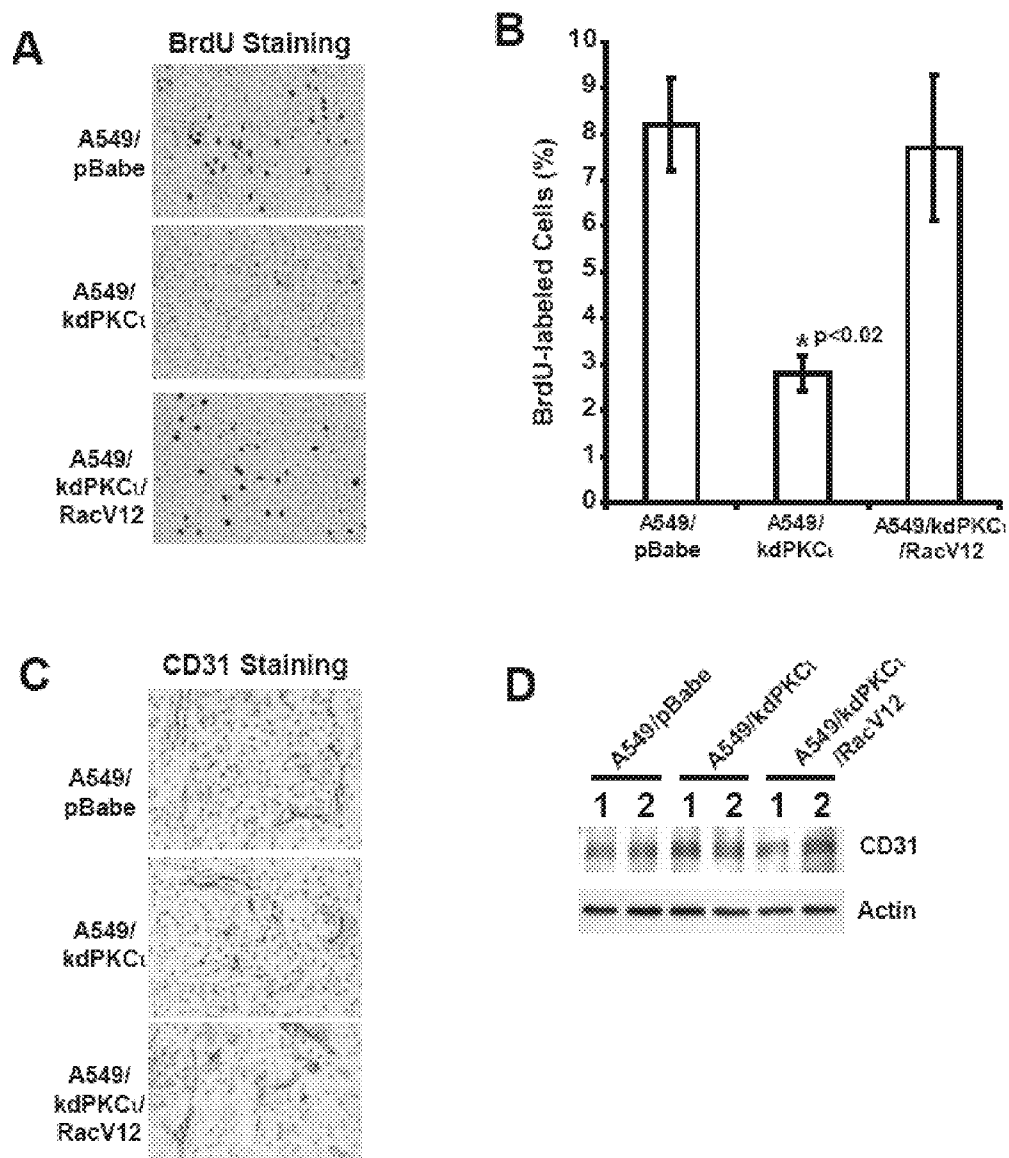

FIG. 10B

FIG. 10C

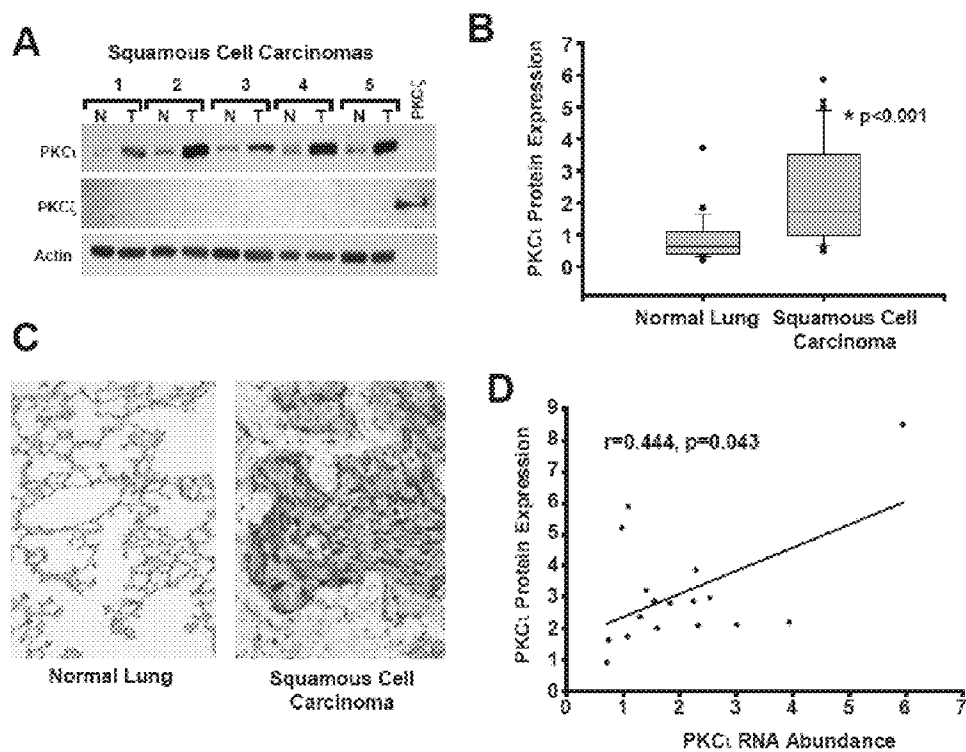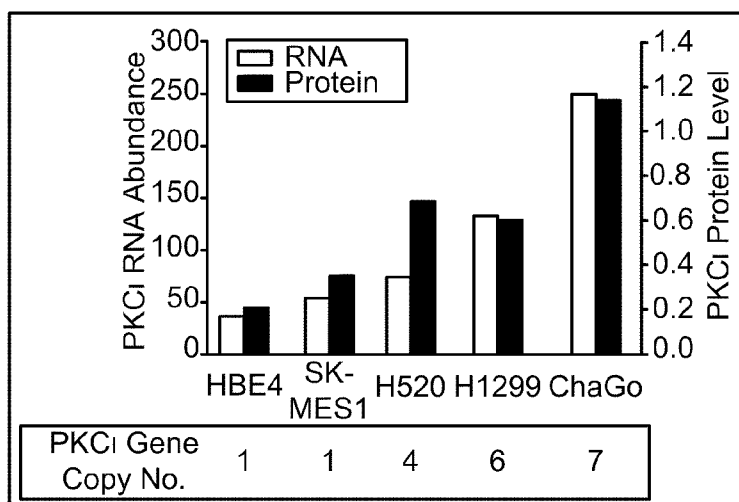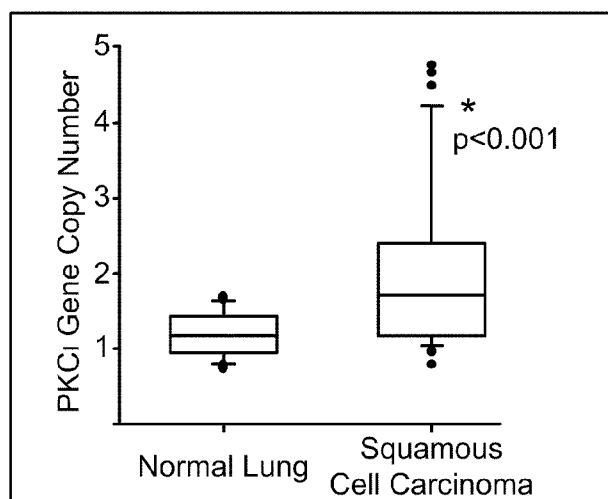FIG. 10D

FIG. 10E

Figure 11

Figure 12

Figure 13

Figure 14

Figure 15

FIG. 16A

FIG. 16B
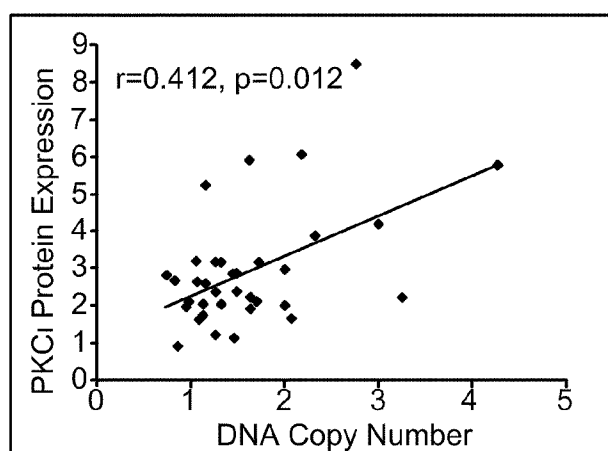
FIG. 16C

Figure 17

Figure 18

Dose Response of ATG and ATM in FRET-based Assay
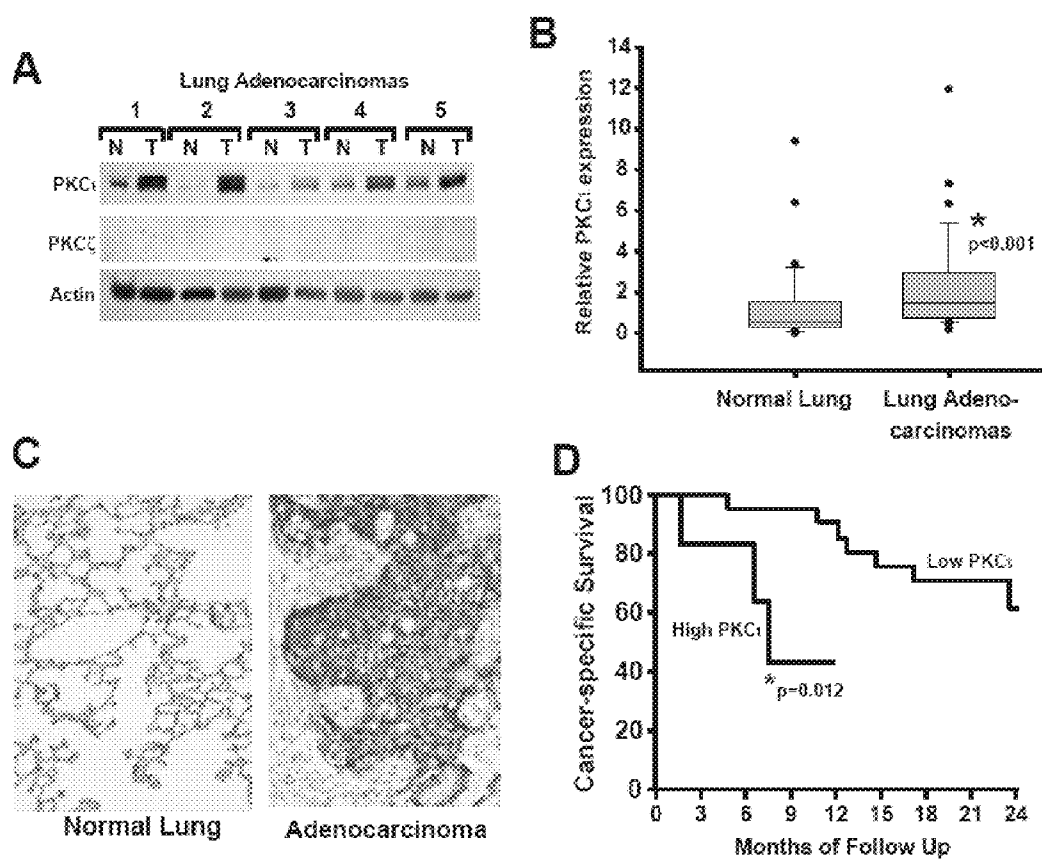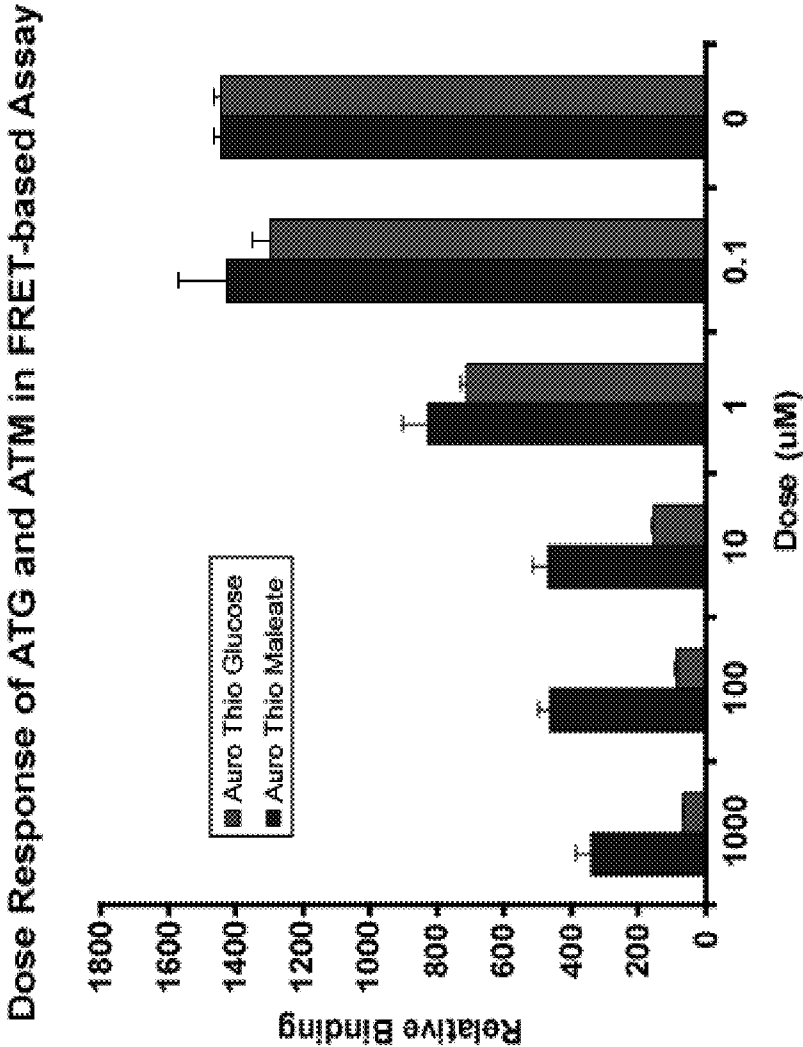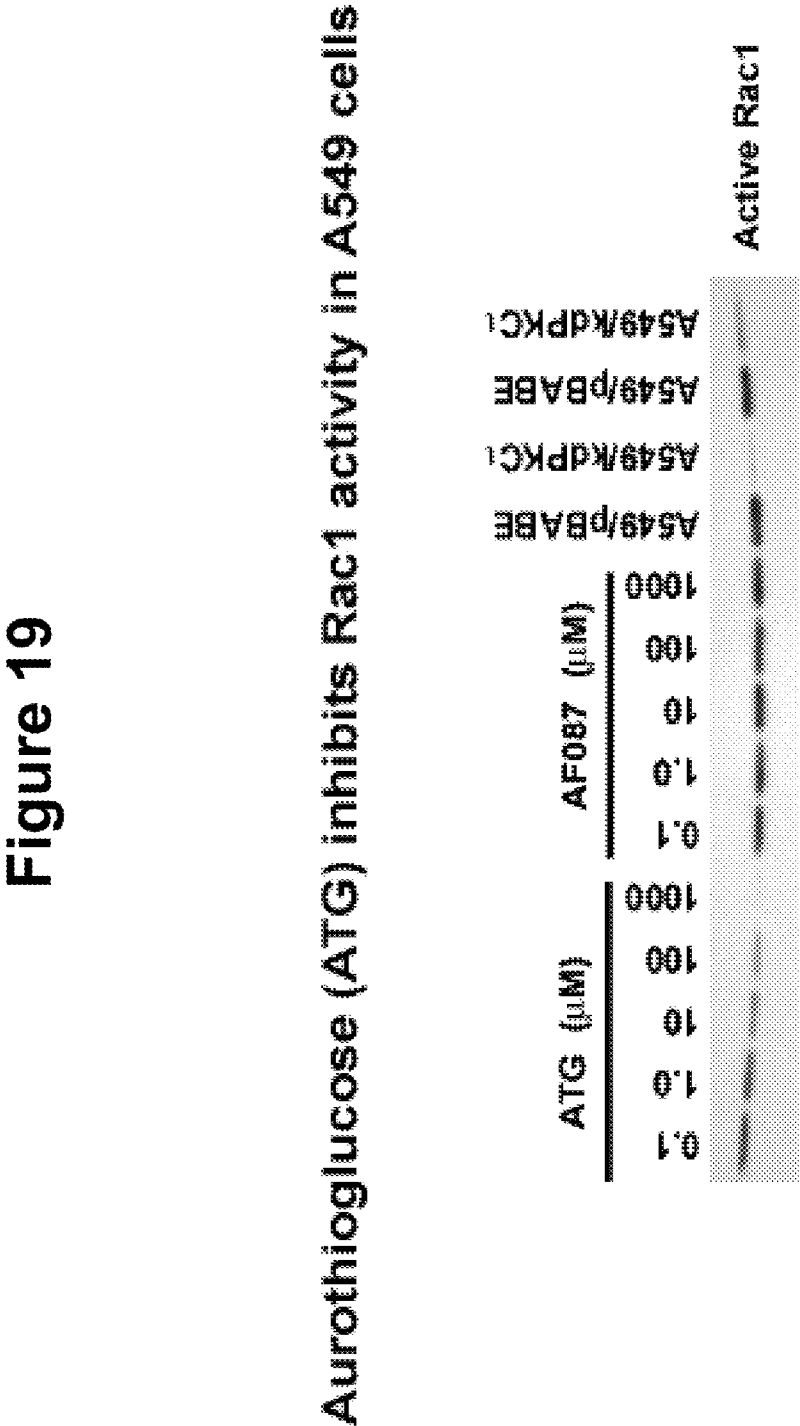
Figure 19

Aurothioglucose (ATG) inhibits Rac1 activity in A549 cells

Figure 20

Figure 21

Effect of ATG on A549 Cell Tumorigenicity in Nude Mice

PROTEIN KINASE C IOTA

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a divisional of U.S. application Ser. No. 10/592,289, filed Jul. 23, 2007, which is a National Stage application under 35 U.S.C. §371 and claims benefit under 35 U.S.C. §119(a) of International Application No. PCT/US2005/007935 having an International Filing Date of Mar. 8, 2005, which claims the benefit of U.S. Provisional Application Ser. No. 60/551,288, filed Mar. 8, 2004.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under 5R01CA081436-09 and 5R01CA094122-06 awarded by The National Institutes of Health National Cancer Institute. The government has certain rights in the invention.

BACKGROUND

[0003] 1. Technical Field
[0004] The invention relates to methods and materials involved in protein kinase C iota signaling. The invention also relates to transgenic animals, inhibitors of protein kinase C iota signaling, methods for inhibiting protein kinase C iota signaling, methods for identifying inhibitors of protein kinase C iota signaling, and methods for diagnosing cancer.
[0005] 2. Background Information
[0006] Protein kinase C iota (PKC iota or PKC_ι) plays a requisite role in Bcr-Abl mediated resistance to chemotherapy-induced apoptosis (Jamieson et al., *J. Biol. Chem.*, 274:3927-3930 (1999) and Murray et al., *J. Biol. Chem.*, 272:27521-4 (1997)), and is critical for epithelial cell polarity (Suzuki et al., *J. Cell Sci.*, 115:3565-73 (2002)) and cell survival (Jamieson et al., *J. Biol. Chem.*, 274:3927-3930 (1999) and Murray et al., *J. Biol. Chem.*, 272:27521-4 (1997)). PKC_ι has also been implicated in Ras-mediated signaling (Coghlan et al., *Mol. Cell. Biol.*, 20:2880-9 (2000); Kampfer et al, *J. Biol. Chem.*, 276:42834-42 (2001); and Uberall et al 1., *J. Cell Biol.*, 144:413-25 (1999)). Activating Ras mutations occur in about 30 percent of all human cancers (Adjei, *J. Natl. Cancer Inst.*, 93:1062-74 (2001)), and in about 50 percent of human colon adenomas and carcinomas (Bos, *Cancer Res.*, 49:4682-9 (1989)). Ras mut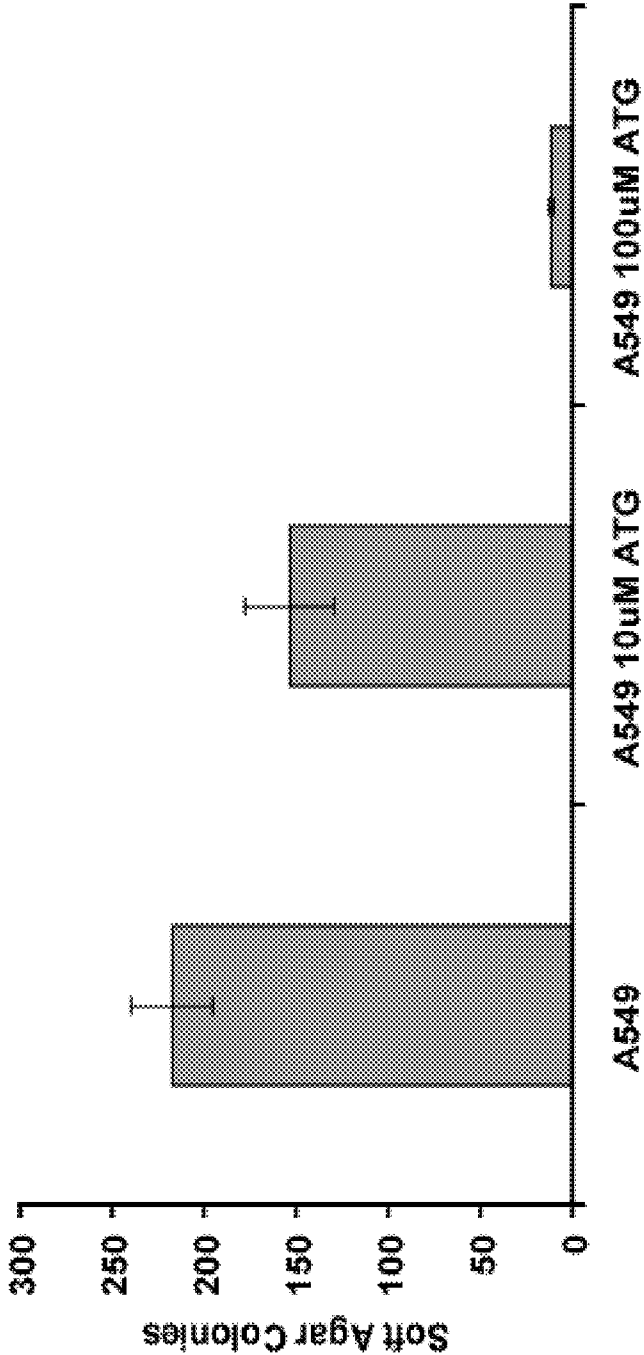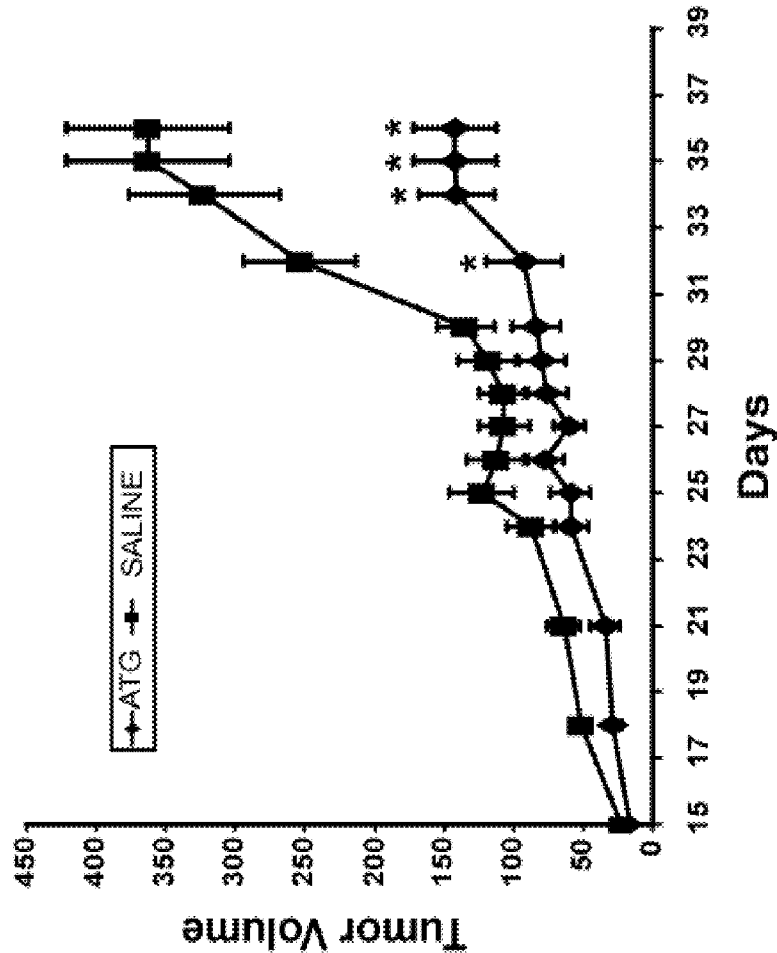ations are an early event in colon carcinogenesis and are often present in preneoplastic lesions in the colon (Pretlow et al., *J. Natl. Cancer Inst.*, 85:2004-7 (1993) and Zaidi et al, *Carcinogenesis.*, 16:451-6 (1995)).

SUMMARY

[0007] The invention involves PKC_ι signaling. The invention relates to transgenic animals, inhibitors of PKC_ι signaling, methods for inhibiting PKC_ι signaling, methods for identifying inhibitors of PKC_ι signaling, and methods for diagnosing cancer. As described herein, Ras-mediated transformation, invasion, and anchorage-independent growth of cells (e.g., intestinal epithelial cells) requires PKC_ι activity. In addition, PKC_ι is involved in Ras- and carcinogen-mediated colon carcinogenesis in vivo. PKC_ι also is involved in other cancers including, without limitation, lung cancers. For example, transgenic mice expressing constitutively active PKC_ι ($caPKC_\iota$) in the colon are highly susceptible to carcinogen-induced colon carcinogenesis, whereas mice expressing

kinase-deficient PKC_ι ($kdPKC_\iota$) are resistant to both carcinogen- and oncogenic Ras-mediated carcinogenesis. Expression of $kdPKC_\iota$ in Ras-transformed rat intestinal epithelial (RIE/Ras) cells blocks oncogenic Ras-mediated activation of Rac1, cellular invasion, and anchorage-independent growth. Constitutively active Rac1 (RacV12) restores invasiveness and anchorage-independent growth in RIE/Ras cells expressing $kdPKC_\iota$. These results demonstrate that PKC_ι is required for oncogenic Ras- and carcinogen-mediated carcinogenesis (e.g., colon carcinogenesis) in vivo and define a pro-carcinogenic signaling axis consisting of Ras, PKC_ι, and Rac1.

[0008] In general, the invention features a transgenic rodent, the nucleated cells of which contain a transgene, the transgene containing a promoter sequence operably linked to a nucleic acid sequence encoding a protein kinase C iota polypeptide, wherein the transgenic rodent expresses the protein kinase C iota polypeptide and develops more preneoplastic colonic lesions after azoxymethane treatment than a corresponding wild-type rodent treated with the azoxymethane. The transgenic rodent can be a mouse. The protein kinase C iota polypeptide can be a constitutively active protein kinase C iota polypeptide. The promoter sequence can promote expression in a cell from the colonic epithelium. The promoter sequence can contain a sequence present in a liver fatty acid-binding protein gene. The promoter sequence can be an $Fabp1^{4x\ at\ -132}$ promoter sequence.

[0009] In another embodiment, the invention features a transgenic rodent, the nucleated cells of which contain a transgene, the transgene containing a promoter sequence operably linked to a nucleic acid sequence encoding a protein kinase C iota polypeptide lacking protein kinase C iota activity, wherein the transgenic rodent expresses the protein kinase C iota polypeptide and exhibits less protein kinase C iota activity in the colonic epithelium than a corresponding wild-type rodent. The transgenic rodent can be a mouse. The promoter sequence can promote expression in a cell from the colonic epithelium. The promoter sequence can contain a sequence present in a liver fatty acid-binding protein gene. The promoter sequence can be a $Fabp1^{4x\ at\ -132}$ promoter sequence. The nucleated cells can contain a second transgene, the second transgene containing a second promoter sequence operably linked to a second nucleic acid sequence encoding a ras polypeptide. The ras polypeptide can be a K-Ras polypeptide. The transgenic rodent can develop fewer aberrant crypt foci in the proximal colon than a corresponding rodent with nucleated cells containing the second transgene and lacking the transgene. The transgenic rodent can be a K-Ras^{LA2}/$kdPKC_\iota$ mouse.

[0010] In another aspect, the invention features progeny of a transgenic rodent, wherein the nucleated cells of the transgenic rodent contain a transgene, the transgene containing (a) a promoter sequence operably linked to a nucleic acid sequence encoding a protein kinase C iota polypeptide, wherein the transgenic rodent expresses the protein kinase C iota polypeptide and develops more preneoplastic colonic lesions after azoxymethane treatment than a corresponding wild-type rodent treated with the azoxymethane, or (b) a promoter sequence operably linked to a nucleic acid sequence encoding a protein kinase C iota polypeptide lacking protein kinase C iota activity, wherein the transgenic rodent expresses the protein kinase C iota polypeptide and exhibits less protein kinase C iota activity in the colonic epithelium than a corresponding wild-type rodent. The nucleated cells of the progeny contain the transgene.

2

[0011] In another aspect, the invention features an isolated cell of a transgenic rodent wherein the nucleated cells of the transgenic rodent contain a transgene, the transgene containing (a) a promoter sequence operably linked to a nucleic acid sequence encoding a protein kinase C iota polypeptide, wherein the transgenic rodent expresses the protein kinase C iota polypeptide and develops more preneoplastic colonic lesions after azoxymethane treatment than a corresponding wild-type rodent treated with the azoxymethane, or (b) a promoter sequence operably linked to a nucleic acid sequence encoding a protein kinase C iota polypeptide lacking protein kinase C iota activity, wherein the transgenic rodent expresses the protein kinase C iota polypeptide and exhibits less protein kinase C iota activity in the colonic epithelium than a corresponding wild-type rodent.

[0012] In another aspect, the invention features a method for inhibiting a protein kinase C iota polypeptide response in a mammal. The method includes administering an inhibitor to the mammal under conditions wherein the response is inhibited, wherein the inhibitor reduces the interaction between a protein kinase C iota polypeptide and a polypeptide selected from the group consisting of Par-6, Src, Par-4, p62/ZIP, and Par-3 polypeptides. The response can be cell transformation, development of cancer, and/or colon carcinogenesis. The inhibitor can be a polypeptide fragment. The polypeptide fragment can contain an amino acid sequence present in the protein kinase C iota polypeptide. The inhibitor can be aurothioglucose, aurothiomaleate, thimerosal, phenylmercuric acetate, ebselen, cisplatin, apomorphine, pyrantel pamoate, gossypol-acetic acid complex, ellagic acid, or hexestrol.

[0013] In another aspect, the invention features a method for identifying an agent that inhibits transformation of a cell. The method includes (a) administering a test agent and a carcinogen to a transgenic rodent, the nucleated cells of which contain a transgene containing a promoter sequence operably linked to a nucleic acid sequence encoding a protein kinase C iota polypeptide, wherein the transgenic rodent expresses the protein kinase C iota polypeptide and develops more preneoplastic colonic lesions after azoxymethane treatment than a corresponding wild-type rodent treated with the azoxymethane, and (b) determining if the test agent inhibits cell transformation in the transgenic rodent as compared with a corresponding transgenic rodent to which the test agent has not been administered. The cell can be an intestinal cell. The test agent can be a test polypeptide. The test polypeptide can contain an amino acid sequence present in a protein kinase C iota polypeptide. The protein kinase C iota polypeptide can be a constitutively active protein kinase C iota polypeptide. The carcinogen can be azoxymethane or dimethylhydrazine.

[0014] In another aspect, the invention features a method for identifying an agent that inhibits the interaction between a protein kinase C iota polypeptide and a polypeptide selected from the group consisting of Par-6, Src, Par-4, p62/ZIP, and Par-3 polypeptides. The method includes (a) contacting a test agent with the protein kinase C iota polypeptide and the polypeptide, wherein the protein kinase C iota polypeptide and the polypeptide each contain a fluorescent molecule under conditions wherein fluorescent resonance energy transfer is detectable when the protein kinase C iota polypeptide interacts with the polypeptide, and (b) determining whether or not the presence of the test agent reduced fluorescent resonance energy transfer between the protein kinase C iota polypeptide and the polypeptide as compared to the fluorescent resonance energy transfer observed between the protein

kinase C iota polypeptide and the polypeptide in the absence of the test agent, wherein a reduction is the fluorescent resonance energy transfer observed between the protein kinase C iota polypeptide and the polypeptide in the presence of the test agent indicates that the test agent is the agent. The polypeptide can be a Par-6 polypeptide. The test agent can be a test polypeptide. The test polypeptide can contain an amino acid sequence present in a protein kinase C iota polypeptide. The test agent can be aurothioglucose, aurothiomaleate, thimerosal, phenylmercuric acetate, ebselen, cisplatin, apomorphine, pyrantel pamoate, gossypol-acetic acid complex, ellagic acid, or hexestrol.

[0015] In another aspect, the invention features a method for determining whether or not a mammal is developing cancerous cells. The method includes determining whether or not the mammal contains an elevated level of a protein kinase C iota polypeptide, wherein the presence of the elevated level of the protein kinase C iota polypeptide indicates that the mammal is developing cancerous cells. The cells can be intestinal cells. The mammal can be a human.

[0016] In another aspect, the invention features a transgenic rodent, the nucleated cells of which contain a transgene. The transgene contains a promoter sequence operably linked to a nucleic acid sequence encoding a protein kinase C iota polypeptide, where the transgenic rodent is capable of expressing the protein kinase C iota polypeptide in lung tissue. The a promoter sequence can be an inducible promoter sequence. The protein kinase C iota polypeptide can be a kinase-deficient protein kinase C iota polypeptide. The carcinogen can be N-nitroso-tris-chloroethylurea. The transgenic rodent can develop more cancerous lesions after carcinogen treatment or expression of a ras polypeptide than a comparable rodent lacking said transgene.

[0017] In another aspect, the invention features a method for inhibiting the binding of a protein kinase C iota polypeptide to a Par-6 polypeptide. The method includes contacting the protein kinase C iota polypeptide or the Par-6 polypeptide with a protein kinase C iota polypeptide/Par-6 polypeptide inhibitor. The protein kinase C iota polypeptide/Par-6 polypeptide inhibitor can be aurothioglucose, aurothiomaleate, thimerosal, phenylmercuric acetate, ebselen, cisplatin, apomorphine, pyrantel pamoate, gossypol-acetic acid complex, ellagic acid, or hexestrol.

[0018] In another aspect, the invention features a method for assessing the prognosis of a mammal (e.g., human) having lung cancer. The method includes determining whether or not the mammal contains cancer cells having an increased copy number of nucleic acid encoding a protein kinase C iota polypeptide or an increased level of protein kinase C iota polypeptide expression or activity, as compared to the copy number or level observed in control cells (e.g., non-cancerous control cells).

[0019] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting. Other fea-

tures and advantages of the invention will be apparent from the following detailed description, and from the claims.

DESCRIPTION OF DRAWINGS

[0020] FIG. 1: PKC_ι expression is increased in azoxymethane (AOM)-induced mouse colon tumors and in human colon tumors. Total protein lysates (a) and total RNA extracts (b) were prepared from AOM-induced mouse colon tumors and uninvolved scraped colonic epithelium from the same animals as described elsewhere (Gokmen-Polar et al., *Cancer Res.,* 61:1375-81 (2001)). (a) Protein extracts were subjected to immunoblot analysis for PKC_ι and actin. (b) Mouse PKC_ι and β-actin mRNA expression was assessed by RT-PCR analysis as described elsewhere (Gokmen-Polar et al., *Cancer Res.,* 61:1375-81 (2001)). Lanes N1-N4, uninvolved mouse colonic epithelium; T1-T4, mouse colon tumors. (c) Total protein lysates were prepared from matched, uninvolved colonic epithelium and colon tumor tissue from five patients with colon carcinoma as described elsewhere (Gokmen-Polar et al., *Cancer Res.,* 61:1375-81 (2001)). Equal amounts of protein (50 µg) were electrophoresed, transferred to nitrocellulose, and subjected to immunoblot analysis for PKC_ι and actin as described elsewhere (Gokmen-Polar et al., *Cancer Res.,* 61:1375-81 (2001)). Lanes N1-N5, uninvolved human colonic epithelium, Lanes T1-T5, matched human colon tumors.

[0021] FIG. 2: PKC_ι expression is elevated in AOM-induced colon tumors. Immunohistochemical analysis of sections from normal, uninvolved epithelium (a and c) and an AOM-induced colon tumor (b and d) in the same animal was performed using a specific PKC_ι antibody in the absence (a and b) or presence (c and d) of a competing PKC_ι peptide. Bars equal 50 µm.

[0022] FIG. 3: Transgenic $caPKC_\iota$ mice are susceptible to AOM-induced colon carcinogenesis. a) and b) Total protein lysates from scraped colonic epithelium from non-transgenic (Ntg) and transgenic a) $caPKC_\iota$ (CA) or b) $kdPKC_\iota$ (KD) mice were subjected to immunoblot analysis for PKC_ι expression (a and b, upper panels) and to immunoprecipitation kinase assay for PKC_ι activity (a and b, lower panels). c) Colons from AOM-treated mice with the indicated genotype were scored for ACF (aberrant crypt foci; McLellan et al., *Carcinogenesis,* 12:2093-8 (1991) and Murray et al., *J. Cell Biol.,* 145:699-711 (1999)). CA/CA: homozygous $caPKC_\iota$ mice; CA/+: heterozygous $caPKC_\iota$ mice, KD/KD: homozygous $kdPKC_\iota$; Ntg: non-transgenic mice. Results represent the average ACF per animal±SEM (n=4-9; *p=0.05 versus Ntg; **p=0.02 versus Ntg). d) H&E stained section of a tubular adenoma from the colon of a non-transgenic mouse 40 weeks after AOM treatment. e) H&E stained section of a carcinoma in situ from the colon of a $caPKC_\iota$ mouse 40 weeks after AOM treatment. d) and e) Bars equal 100 µm.

[0023] FIG. 4: PKC_ι is required for oncogenic Ras-induced Rac1 activation and invasion in vitro. a) rat intestinal epithelial (RIE) cells were stably transfected with control empty vector (RIE); Ras (RIE/Ras); Ras and $wtPKC_\iota$ (RIE/Ras/$wtPKC_\iota$); or Ras and $dnPKC_\iota$ (RIE/Ras/$kdPKC_\iota$). Total cell lysates from these cell lines were subjected to immunoblot analysis for expression of PKC_ι (first panel), oncogenic V12 Ras (second panel) and β-actin (third panel). Immunoprecipitates from cells using a specific PKC_ι antibody were analyzed by immunoblot analysis for PKC_ι (fourth panel) and for PKC_ι activity (fifth panel). Anti-FLAG immunoprecipitates from these cells were analyzed by immunoblot analysis for PKC_ι

(sixth panel) and assayed for PKC_ι activity (seventh panel). b) Growth of RIE cells and RIE cell transfectants was monitored daily by measuring OD_{570} after reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide as described previously (Murray et al., *J. Cell Biol.,* 157:915-920 (2002)). Data represent the mean±SD from three independent determinations. c) Active (GTP-bound) Rac1 was isolated from the indicated RIE cell transfectants: control empty vector; Ras; Ras and a dominant negative Rac1 (RacN17); Ras and $kdPKC_\iota$; and Ras and $kdPKC_\iota$ and RacV12. Immunoblot analysis was conducted for active Rac1 (upper panel), total cellular Rac1 (middle panel) and β-actin (lower panel). The asterisk indicates the migration of Myc-tagged, virally-expressed Rac1 mutants. d) The indicated RIE transfectants were evaluated for invasiveness in Matrigel-coated Transwell chambers. Data represent the average number of cells invading into the lower chamber±SD from three independent experiments. *p=0.02 versus RIE+control vector; **p=or <0.02 versus RIE/Ras; ***p=0.005 versus RIE/Ras/$kdPKC_\iota$.

[0024] FIG. 5: Expression of $dnPKC_\iota$ blocks Ras-mediated transformation of the intestinal epithelium in vitro and in vivo. a) and b) RIE cells were stably transfected with control empty vector (RIE), Ras (RIE/Ras), Ras and $wtPKC_\iota$ (RIE/Ras/$wtPKC_\iota$), or Ras and $kdPKC_\iota$ (RIE/Ras/$kdPKC_\iota$) and evaluated for growth in soft agar. Colonies were visualized by staining with Giemsa and enumerated. a) Representative experimental results. Numbers in parenthesis represents number of colonies formed in each dish. b) Values represent the average of three independent soft agar colony formation experiments ±SEM. *p<0.002 versus RIE/Ras. c) The indicated RIE cell transfectants were analyzed as described in a). Values represent the average of five determinations±SEM. *p=0.008 versus RIE/Ras; **p=0.0001 versus RIE/Ras/kdP-KC_ι. d) Twelve week old $K\text{-}Ras^{LA2}$ and $K\text{-}Ras^{LA2}$/kd PKC_ι mice were analyzed for ACF in the proximal colon. Average number of ACF per mouse is plotted +/-the SEM, (n=5)*p=0.04.

[0025] FIG. 6 is a listing of a nucleic acid sequence (SEQ ID NO:1) that encodes an amino acid sequence (SEQ ID NO:2) of a constitutively active mutant of human PKC_ι. The mutation is highlighted in underlined bold.

[0026] FIG. 7 is a listing of a nucleic acid sequence (SEQ ID NO:3) that encodes an amino acid sequence (SEQ ID NO:4) of a kinase deficient mutant of human PKC_ι. The mutation is highlighted in underlined bold.

[0027] FIG. 8: Expression of PKC_ι in Human Cancer Tissues. A). Immunoblot analysis of PKC_ι expression in human non-small lung cancer cell lines. HBE4 is a non-transformed human lung epithelial cell line. The other cell lines are established human lung cancer cell lines obtained from ATCC. B) Immunoblot analysis of non-small cell lung cancer patient samples and matched normal lung epithelium from 7 patients. N=normal lung epithelium; T=lung tumor tissue from same patient. 1-7=case number. Cases 1-4 are on one immunoblot, and cases 5-7 are on a separate blot. C) Immunohistochemical staining of sections from normal lung epithelium, an adenocarcinoma of the lung, and a squamous cell carcinoma of the lung. These results are representative of more than 80 lung cancer patient samples examined.

[0028] FIG. 9 is a bar graph plotting the number of soft agar colonies by RIE/Ras cells and RIE/Ras/PKC_ι (1-113) cells. The cells with PKC_ι (1-113) exhibited significantly less soft

agar colony formation than cells lacking PKC$_ι$ (1-113). These results demonstrate that PKC$_ι$ (1-113) can block Ras transformation.

[0029] FIG. 10A contains a photograph of an immunoblot analysis of human NSCLC cell lines for PKC$_ι$, PKCζ, and actin. PKC$_ι$ is overexpressed in all NSCLC cell lines, whereas PKCζ was not detected. Purified recombinant human PKCζ and PKC$_ι$ were included as controls for antibody specificity. FIG. 10B contains a photograph of an immunoblot analysis of A549 cell transfectants expressing either pBabe, wild-type human PKC$_ι$ (wtPKC$_ι$), or kinase-deficient human PKC$_ι$ (kd-PKC$_ι$) for Flag, PKC$_ι$, and actin. FIG. 10C is a graph plotting growth of A549 transfectants in adherent culture in growth medium supplemented with 10%, 2%, or no serum. FIG. 10D is a bar graph plotting the number of A549/pBabe, A549/wtPKC$_ι$, and A549/kdPKC$_ι$ cells invading through Matrigel coated chambers. FIG. 10E is a bar graph plotting the number of colonies formed by anchorage-independent growth of A549/pBabe, A549/wtPKC$_ι$, or A549/kdPKC$_ι$ cells in soft agar.

[0030] FIG. 11A contains a photograph of an immunoblot analysis of H1299 cells stably transfected with pBabe, kdP-KC$_ι$, or wtPKC$_ι$ for Flag, PKC$_ι$, or actin. FIG. 11B is a graph plotting the growth kinetics of H1299 cell transfectants in adherent culture in the presence of 10% serum. FIG. 11C is a bar graph plotting the number of H1299 cell tranfectants invading through Matrigel coated chambers. FIG. 11D is a bar graph plotting the number of colonies formed by anchorage-independent growth of H1299 cell transfectants in soft agar. FIG. 11E contains a photograph of an immunoblot analysis of ChaGoK cells stably transfected with empty pBabe or kdP-KC$_ι$ for Flag, PKC$_ι$, and actin.

[0031] FIG. 11E also contains a bar graph plotting the number of colonies formed by anchorage-independent growth of ChaGoK cell transfectants in soft agar.

[0032] FIG. 12A contains a photograph of active GTP-bound Rac1 and total Rac1 expression in A549 and H1299 cell transfectants. FIG. 12B contains a photograph of an immunoblot analysis of A549 and H1299 cell transfectants for cIAP2, Bcl-XL, and actin.

[0033] FIG. 12C contains a photograph of the analysis of A549 and H1299 cell transfectants for PARP and cleaved PARP. HeLa cells treated with taxol for either 24 or 48 hours served as a positive control. FIG. 12D is a bar graph plotting transcriptional activity of an NF-κB-luciferase reporter in A549/pBabe and A549/kdPKC$_ι$ cells in the presence and absence of TNFα. FIG. 12E contains a photograph of active and total Rac1 expression assessed in parental A549 cells, A549 cells treated with the PKC$_ι$-selective pseudosubstrate peptide inhibitor (PSI), and A549 cells expressing the PB1 domain of PKC$_ι$ (PKC$_ι$ (1-113)). FIG. 12F is a bar graph plotting the number of A549/LZRS and A549/PKC$_ι$ (1-113) cell transfectants invading through Matrigel coated chambers. FIG. 12G is a bar graph plotting the number of colonies formed by anchorage-independent growth of A549/LZRS and A549/PKC$_ι$ (1-113) cell transfectants in soft agar.

[0034] FIG. 13A is a bar graph plotting the number of colonies formed by anchorage-independent growth of A549/pBabe, A549/kdPKC$_ι$, and A549/kdPKC$_ι$/RacV12 cell transfectants in soft agar. FIG. 13B is a graph plotting the tumorigenic growth of A549 cell transfectants as subcutaneous xenografts in nude mice. FIG. 13C is a photograph of an immunoblot analysis of A549 cell transfectants grown as xenografts in nude mice for phospho-Ser217/221 MEK,

phospho-Ser298 MEK, total MEK, phosphor-Thr202/Tyr204 ERK 1/2, and total ERK 1/2. FIG. 13D contains a photograph of an immunoblot analysis of A549 transfectants grown as xenografts in nude mice for Bcl-XL, cIAP2, actin, and PARP/cleaved PARP. Taxol-treated HeLa cells were included as a positive control for cleaved PARP.

[0035] FIG. 14A contains photographs of immunohistochemical staining of A549/pBabe, A549/kdPKC$_ι$, and A549/kdPKC$_ι$/RacV12 cell tumors for BrdU. Tumor-bearing animals were injected intraperitoneally with BrdU one hour prior to sacrifice. FIG. 14B is a bar graph plotting the percent of BrdU-labeled cells in A549/pBabe, A549/kdPKC$_ι$, and A549/kdPKC$_ι$/RacV12 tumors. FIG. 14C contains photographs of immunohistochemical staining of A549 cell transfectant tumors for the endothelial cell marker, CD31. FIG. 14D contains a photograph of an immunoblot analysis of A549 cell transfectant tumors for CD31 and actin.

[0036] FIG. 15A contains a photograph of immunoblot analysis of primary SCCs and matched normal lung tissue for PKC$_ι$, PKCζ, and actin. FIG. 15B is a bar graph plotting PKC$_ι$ expression in primary SCCs. 36 cases of primary SCC and matched normal lung tissue were analyzed. FIG. 15C contains photographs of immunohistochemistry of normal lung and SCC for PKC$_ι$. FIG. 15D is a graph plotting the correlation between PKC$_ι$ mRNA abundance and PKC$_ι$ polypeptide expression in SCC.

[0037] FIG. 16A is a graph of an analysis of human SCC cell lines for PKC$_ι$ gene copy number, mRNA abundance, and polypeptide expression. FIG. 16B is a graph plotting PKC$_ι$ gene copy number for normal lung and primary squamous cell carcinomas. FIG. 16C is a graph plotting the correlation between PKC$_ι$ expression and PKC$_ι$ gene copy number.

[0038] FIG. 17A contains a photograph of an immunoblot analysis of primary lung adenocarcinomas and matched normal lung tissue for PKC$_ι$, PKCζ, and actin. FIG. 17B is graph plotting PKC$_ι$ expression in normal lung and primary lung adenocarcinomas. 36 cases of primary LAC and matched normal lung tissue were analyzed. FIG. 17C contains photographs of immunohistochemistry of normal lung and lung adenocarcinoma for PKC$_ι$. FIG. 17D is a graph plotting the Kaplan-Meier survival curve for LAC expressing low versus high PKC$_ι$. PKC$_ι$ expression correlates with poor survival.

[0039] FIG. 18 is a bar graph plotting the dose response of ATG and ATM in a FRET-based assay designed to assess the interaction between PAR6 and PCK$_ι$ polypeptides.

[0040] FIG. 19 contains a photograph of an immunoblot analysis of Rac1 activity in cells treated with ATG.

[0041] FIG. 20 is a bar graph plotting the number of colonies formed by anchorage-independent growth of A549 cells in soft agar. The cells were either untreated or treated with ATG (10 μM or 100 μM).

[0042] FIG. 21 is a graph plotting tumor volume for mice treated with saline or ATG.

DETAILED DESCRIPTION

[0043] The invention provides methods and materials related to PKC$_ι$ signaling. It is noted that PKC$_ι$ generally refers to a human polypeptide. The corresponding polypeptide in rodents, which is about 95 percent homologous at the amino acid level to human PKC$_ι$, is generally referred to as protein kinase C lambda. For the purpose of this document, the term "PKC$_ι$" refers to any PKC$_ι$ polypeptide including, without limitation, human PKC$_ι$ polypeptides and rodent protein kinase C lambda.

5

[0044] In some embodiments, the invention provides transgenic non-human animals. Such non-human animals can be farm animals such as pigs, goats, sheep, cows, horses, and rabbits, rodents such as rats, guinea pigs, and mice, and non-human primates such as baboons, monkeys, and chimpanzees. The term "transgenic non-human animal" as used herein includes, without limitation, founder transgenic non-human animals as well as progeny of the founders, progeny of the progeny, and so forth, provided that the progeny retain the transgene. The nucleated cells of the transgenic non-human animals provided herein can contain a transgene that includes a promoter sequence operably linked to a nucleic acid sequence encoding a PKC$_\iota$ polypeptide. A PKC$_\iota$ polypeptide can be a wild-type PKC$_\iota$ polypeptide (e.g., wild-type human PKC$_\iota$), a constitutively active PKC$_\iota$ polypeptide (e.g., constitutively active human PKC$_\iota$), or a kinase deficient PKC$_\iota$ polypeptide (e.g., kinase deficient human PKC$_\iota$). The transgenic non-human animal can express the PKC$_\iota$ polypeptide and can develop more preneoplastic colonic lesions after carcinogen (e.g., azoxymethane) treatment than a corresponding wild-type non-human animal treated with the carcinogen.

[0045] The nucleic acid sequence encoding the PKC$_\iota$ polypeptide can be a cDNA or can include introns or adjacent 5'- or 3'-untranslated regions (e.g., a genomic nucleic acid). The nucleic acid sequence encoding the PKC$_\iota$ polypeptide can be operably linked to any promoter sequence. For example, a promoter sequence that facilitates the expression of a nucleic acid without significant tissue- or temporal-specificity can be used. Examples of such promoter sequences include, without limitation, viral promoters such as a herpes virus thymidine kinase (TK) promoter sequence, a SV40 promoter sequence, or a cytomegalovirus (CMV) promoter sequence. In some examples, nucleic acid encoding a PKC$_\iota$ polypeptide can be operably linked to a tissue-specific promoter sequence such as a colon-specific promoter sequence (e.g., a Fabp1$^{4x\,at\,-132}$ promoter sequence). Other tissue-specific promoter sequences include, without limitation, those listed in Table 1.

TABLE 1

Alternative Tissue-specific promoters

Tissue	Promoter	Reference
Breast	Whey acidic protein	Tessier et al., Cancer Res., 64: 209-214 (2004)
Prostate	Probasin	Masumori et al., Cancer Res., 61: 2239-2249 (2001)
Lung	Surfactant protein C	Glasser et al., Am. J. Physiol. Lung Cell Mol. Physiol., 278: L933-945 (2000)
Kidney	Ksp-cadherin	Igarashi et al., Am. J. Physiol, 277: F599-610 (1999)
Liver	Albumin	Pinkert et al., Genes Dev., 1: 268-276 (1987)
Brain	Glial fibrillary acidic protein	Shi et al., Proc. Natl. Acad. Sci. USA, 98: 12754-12759 (2001)
Pancreas	Human pancreatic secretory trypsin inhibitor	Yasuda et al., J. Biol. Chem., 273: 34413-34421 (1998)

[0046] In some cases, an inducible promoter sequence can be used. For example, a Tet-on or Tet-off expression system can be used to design one or more constructs that allow expression to be regulated in response to a drug (e.g., tetracycline or doxycycline). Briefly, Tet-on and Tet-off expression systems are binary transgenic systems in which expression from a target transgene depends on the activity of an inducible transcriptional activator. In both the Tet-on and Tet-off systems, expression of the transcriptional activator can be regulated both reversibly and quantitatively by exposing a transgenic animal to varying concentrations of tetracycline or a tetracycline derivatives such as doxycycline. In some cases, a Tet-on or Tet-off system can be used with a tissue-specific promoter sequence (e.g., a lung-specific promoter sequence) such that a PKC$_\iota$ polypeptide is expressed in a particular tissue (e.g., lung tissue) in response to changes in, for example, tetracycline or doxycycline.

[0047] The term "operably linked" as used herein refers to positioning a regulatory element (e.g., a promoter sequence, an inducible element, or an enhancer sequence) relative to a nucleic acid sequence encoding a polypeptide in such a way as to permit or facilitate expression of the encoded polypeptide. In the transgenes disclosed herein, for example, an enhancer can be positioned 3' or 5' relative to the nucleic acid encoding a PKC$_\iota$ polypeptide, and can be positioned within the transgene in either the 5' to 3' or the 3' to 5' orientation.

[0048] Various techniques known in the art can be used to introduce transgenes into non-human animals to produce founder lines, in which the transgene is integrated into the genome. Such techniques include, without limitation, pronuclear microinjection (See, e.g., U.S. Pat. No. 4,873,191), retrovirus mediated gene transfer into germ lines (Van der Putten et al., *Proc. Natl. Acad. Sci. USA,* 82:6148-1652 (1985)), gene targeting into embryonic stem cells (Thompson et al., Cell 56:313-321 (1989)), electroporation of embryos (Lo, *Mol. Cell. Biol.,* 3:1803-1814 (1983)), and in vitro transformation of somatic cells, such as cumulus or mammary cells, followed by nuclear transplantation (Wilmut et al., *Nature,* 385:810-813 (1997); and Wakayama et al., *Nature,* 394:369-374 (1998)). For example, fetal fibroblasts can be genetically modified to express a PKC$_\iota$ polypeptide, and then fused with enucleated oocytes. After activation of the oocytes, the eggs are cultured to the blastocyst stage. See, for example, Cibelli et al., *Science,* 280:1256-1258 (1998). Standard breeding techniques can be used to create animals that are homozygous for the transgene from the initial heterozygous founder animals. Homozygosity is not required, however, as the phenotype can be observed in hemizygotic animals.

[0049] Once transgenic non-human animals have been generated, expression of a PKC$_\iota$ polypeptide can be assessed using standard techniques. Initial screening can be accomplished by Southern blot analysis to determine whether or not integration of the transgene has taken place. For a description of Southern analysis, see sections 9.37-9.52 of Sambrook et al., 1989, *Molecular Cloning, A Laboratory Manual,* second edition, Cold Spring Harbor Press, Plainview; NY. Polymerase chain reaction (PCR) techniques also can be used in the initial screening. PCR refers to a procedure or technique in which target nucleic acids are amplified. Generally, sequence information from the ends of the region of interest or beyond is employed to design oligonucleotide primers that are identical or similar in sequence to opposite strands of the template to be amplified. PCR can be used to amplify specific sequences from DNA as well as RNA, including sequences from total genomic DNA or total cellular RNA. Primers typically are 14 to 40 nucleotides in length, but can range from 10 nucleotides to hundreds of nucleotides in length. PCR is described in, for example *PCR Primer: A Laboratory Manual,* ed. Dieffenbach and Dveksler, Cold Spring Harbor

6

Laboratory Press, 1995. Nucleic acids also can be amplified by ligase chain reaction, strand displacement amplification, self-sustained sequence replication, or nucleic acid sequence-based amplified. See, for example, Lewis, *Genetic Engineering News,* 12:1 (1992); Guatelli et al., *Proc. Natl. Acad. Sci. USA,* 87:1874-1878 (1990); and Weiss, *Science,* 254:1292-1293 (1991).

[0050] Expression of a nucleic acid sequence encoding a PKC_ι polypeptide in the tissues of transgenic non-human animals can be assessed using techniques that include, without limitation, Northern blot analysis of tissue samples obtained from the animal (e.g., intestinal tissue), in situ hybridization analysis, Western analysis, immunoassays such as enzyme-linked immunosorbent assays, and reverse-transcriptase PCR(RT-PCR). As described herein, expression of a constitutively active PKC_ι polypeptide can result in transgenic animals exhibiting more preneoplastic colonic lesions after carcinogen (e.g., azoxymethane) treatment than a corresponding wild-type animal treated with the carcinogen.

[0051] In some embodiments, transgenic animals containing a transgene that encodes a PKC_ι polypeptide lacking protein kinase C iota activity can exhibit less protein kinase C iota activity in, for example, the colonic epithelium than a corresponding wild-type rodent. It is understood that a particular phenotype in a transgenic animal typically is assessed by comparing the phenotype in the transgenic animal to the corresponding phenotype exhibited by a control non-human animal that lacks the transgene.

[0052] In some embodiments, the transgenic non-human animals can include a second transgene that contains a nucleic acid sequence of an oncogene such as ras (e.g., a K-Ras polypeptide). The nucleic acid sequence of human K-ras can be obtained from GenBank (e.g., GenBank Accession No. NM_033360). The second transgene also can include regulatory elements as discussed above (e.g., a tissue-specific promoter sequence).

[0053] The invention also provides tissues (e.g., colon sections, lung sections, etc.) and cells (e.g., intestinal cells, lung cells, etc.) obtained from the transgenic non-human animals provided herein.

[0054] In addition, the invention provides inhibitors of PKC_ι signaling. For example, a polypeptide sequence corresponding to amino acids 1-113 of a PKC_ι polypeptide can be used to block Ras-mediated transformation. Expression of the 1-113 polypeptide region of PKC_ι appears to block PKC_ι signaling through disruption of protein/protein interactions between PKC_ι and Par-6. Polypeptides shorter (e.g., the 1-110 region, the 5-113 region, the 10-113 region, or 5-110 region) or longer (e.g., the 1-115 region, the 1-117 region, or the 1-120 region) than a 113 amino acid fragment of a PKC_ι polypeptide can be used as an inhibitor of PKC_ι signaling.

[0055] In addition, polypeptides derived from other regions of PKC_ι that are involved in the interaction of PKC_ι with other signaling molecules (e.g., Src, Par-4, p62/ZIP, and Par-3 polypeptides) can be used as inhibitors of PKC_ι signaling. Likewise, the corresponding regions on molecules such as Par-6, Src, Par-4, p62/ZIP, and Par-3 that mediate the binding of these molecules to PKC_ι can be used as inhibitors. Regions that can be used to design an inhibitor include, without limitation, (a) the MOO domain that mediates binding of Src to PKC_ι and (b) sites on PKC_ι that are phosphorylated (either by PKC_ι itself or by other kinases). For example, Src phosphorylates multiple sites on PKC_ι including tyrosines 256, 271 and 325 (Wooten et al., *Mol. Cell. Biol.,* 21:8414-8427

(2001)). Phosphorylation at Y325 can be responsible for src-mediated activation of PKC_ι activity. Polypeptides surrounding this region can act as inhibitors of src-mediated activation of PKC_ι. Likewise, phosphorylation of Y256 (by src or other kinases) can regulate the ability of PKC_ι to enter the nucleus of the cell (White et al., *J. Cell. Biochem.,* 85:42-53 (2002)), although other regions on PKC_ι can also be involved in regulating nuclear localization of PKC_ι (Perander et al., *J. Biol. Chem.,* 276:13015-13024 (2001)). Expression of polypeptides surrounding any of these regions of PKC_ι can be used to disrupt PKC_ι signaling.

[0056] In some embodiments, an inhibitor of PKC_ι signaling is not a polypeptide. Examples of non-polypeptide inhibitors of PKC_ι signaling include, without limitation, aurothioglucose, aurothiomaleate, thimerosal, phenylmercuric acetate, ebselen, cisplatin, apomorphine, pyrantel pamoate, gossypol-acetic acid complex, ellagic acid, and hexestrol.

[0057] The invention also provides methods for identifying PKC_ι signaling inhibitors. In general, such methods include (a) designing an assay to measure the binding of a PKC_ι polypeptide and a polypeptide (e.g., a Par6 polypeptide) that interacts with a PKC_ι polypeptide and (b) screening for compounds that disrupt this interaction. For example, expression plasmids can be designed to express a fragment of a Par6 polypeptide (e.g. amino acids 1-125 of a human Par6 polypeptide) as a fusion protein containing a naturally fluorescent protein (e.g., cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP)). Another set of plasmids can be designed to express a region of a PKC_ι polypeptide (e.g., amino acids 1-113 or a full-length PKC_ι polypeptide) that binds to the Par6 region. This region of a PKC_ι polypeptide also can be expressed as a fusion protein with either CFP or YFP. The binding of these recombinant polypeptides can be followed by measuring fluorescence from the polypeptides when the complex is excited by a specific wave length of light. CFP and YFP fluoresce when they are stimulated by light. However, the wavelength of light that excites CFP is different from that which excites YFP. Thus, if one wavelength of light is used, CFP can emit cyan fluorescent light but YFP will not fluoresce. If a different wavelength of light is used, YFP can fluoresce yellow, but CFP will not fluoresce. When CFP and YFP are brought into very close proximity, such as when Par6/CFP and PKC_ι/YFP bind to each other, and when the wavelength of light is used that will cause CFP to emit cyan fluorescent light, then some of the energy that would ordinarily be emitted as cyan colored light will be transferred to the adjacent YFP molecule on the PKC_ι/YFP molecule. This energy can excite YFP to emit yellow fluorescent light. This process of energy transfer from CFP to YFP is called fluorescence energy transfer (FRET). FRET can be a very sensitive way of measuring binding between two molecules that contain CFP and YFP. For example, when Par6/CFP and PKCl/YFP (or the converse pair: Par6/YFP and PKC_ι/CFP) are put together, FRET can occur. In addition, FRET can be used to assess binding of these two molecules since when binding is disrupted, FRET can be abolished.

[0058] In one embodiment, recombinant Par6/CFP and PKC_ι/YFP polypeptides can be added to the wells of either 96 well or 384 well plates. Then, a single compound from a large compound library can be added to each of the individual wells. The entire plate can be placed in a fluorescence plate reader that can measure FRET in each of the wells. Those wells that show a decrease or loss of FRET can contain a compound that can potentially disrupt the interaction

between Par6 and PKC$_\iota$. Appropriate controls can be included in the assay to avoid identifying compounds that inhibit FRET by other, non-specific means. This type of assay can be adapted for high throughput screening of compound libraries containing thousands and even hundreds of thousands of compounds.

[0059] Once a compound is identified as being a candidate for disrupting the interaction of Par6 and PKC$_\iota$ polypeptides, the compound can be put through a secondary screen in which its ability to disrupt Par6/PKC$_\iota$ polypeptide binding is determined in cells expressing recombinant Par6 and PKC$_\iota$ polypeptides. Compounds that disrupt Par6/PKC$_\iota$ polypeptide binding in cells can be further screened for the ability to inhibit PKC$_\iota$-dependent cellular transformation.

[0060] The invention also provides methods for diagnosing cancer. For example, samples can be obtained and assessed for the presence of an elevated level of PKC$_\iota$ polypeptides or an elevated level of PKC$_\iota$ polypeptide activity. The presence of an elevated level of PKC$_\iota$ polypeptides or elevated level of PKC$_\iota$ polypeptide activity can indicate the presence of cancer and/or precancerous cells. Any method can be used to assess the level of PKC$_\iota$ polypeptide expression. For example, immunoblot analysis and/or immunohistochemistry can be used to examine the expression of PKC$_\iota$ polypeptides in tissue and/or cell samples. In some cases, PKC$_\iota$ polypeptide activity can be assessed using any of the methods provided herein.

[0061] The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1

Methods and Materials

Analysis of PKC$_\iota$ Expression in Mouse and Human Colon Tumors

[0062] AOM-induced colon tumors were produced in C57B1/6 mice as described elsewhere (Gokmen-Polar et al., *Cancer Res.*, 61:1375-81 (2001)). Fresh frozen tissue from human colon carcinomas and uninvolved colonic epithelium was obtained from surgical specimens. Isolation of RNA and protein for RT-PCR and immunoblot analysis, respectively, was performed as described elsewhere (Gokmen-Polar et al., *Cancer Res.*, 61:1375-81 (2001)). Immunoblot analysis for PKC$_\iota$ and actin was conducted using isozyme-specific antibody against PKC$_\iota$ and actin (Santa Cruz, Inc.) as described elsewhere (Gokmen-Polar et al., *Cancer Res.*, 61:1375-81 (2001) and Murray et al., *J. Biol. Chem.*, 272:27521-4 (1997)). This PKC$_\iota$ antibody recognizes PKC$_\iota$, but not PKCζ (Murray et al., *J. Biol. Chem.*, 272:27521-4 (1997)).

[0063] Primers for RT-PCR analysis were as follows: PKC$_\iota$ forward primer: 5'-GCTTA-TGTTTGAGATGATGGCGG-3' (SEQ ID NO:5), PKC$_\iota$ reverse primer: 5-GTGACA-AC-CCAATCGTTCCG-3' (SEQ ID NO:6); actin forward primer: 5'-GTGGGC-CGCTCTAGGCACCAA-3' (SEQ ID NO:7), actin reverse primer: 5'-CTCTTTGAT-GTCACG-CACGATTTC-3' (SEQ ID NO:8).

[0064] Colon tumors and uninvolved colonic epithelium from AOM-treated mice were fixed in 10% buffered formalin, sectioned, and subjected to antigen retrieval (Vector Labs). Immunohistochemical detection of PKC$_\iota$ was performed using the specific PKC$_\iota$ antibody (Santa Cruz) and the DAKO

LSAB2 (DAB) detection system (DAKO). Specificity of immunostaining for PKC$_\iota$ was demonstrated by inclusion of a 5-fold molar excess of the peptide used to generate the PKC$_\iota$ antibody (Santa Cruz) in the antibody dilution. Digital images were acquired on an Olympus DX51 microscope equipped with a DP70 digital camera using a 20× objective lens. Images were captured using the DP Controller software and processed in Adobe Photoshop.

Production of Transgenic Mice and Carcinogenesis Studies

[0065] Nucleic acid encoding a constitutively active human PKC$_\iota$ (caPKC$_\iota$) and nucleic acid encoding a kinase deficient human PKC$_\iota$ (kdPKC$_\iota$) were generated and characterized elsewhere (Jamieson et al., *J. Biol. Chem.*, 274:3927-3930 (1999) and Lu et al., *Oncogene*, 20:4777-4792 (2001)). Transgenic caPKC$_\iota$ and kdPKC$_\iota$ mice were generated on a C57B1/6 background using the Fabp1$^{4x\ at\ -132}$ promoter (Simon et al., *J. Biol. Chem.*, 272:10652-63 (1997); provided by J. Gordon, Washington University, St. Louis, Mo.) to direct transgene expression to the colonic epithelium (Murray et al., *J. Cell Biol.*, 145:699-711 (1999)). Isolation of colonic epithelium, immunoblot analysis for PKC$_\iota$, and immunoprecipitation histone kinase assays were described elsewhere (Jamieson et al., *J. Biol. Chem.*, 274:3927-3930 (1999) and Murray et al., *J. Cell Biol.*, 145:699-711 (1999)). Transgenic caPKC$_\iota$, transgenic kdPKC$_\iota$, and non-transgenic mice were injected with either AOM (10 mg/kg) or saline as described elsewhere (Gokmen-Polar et al., *Cancer Res.*, 61:1375-81 (2001)). ACF analysis was performed 12 weeks after the last AOM injection (Murray et al., *J. Cell Biol.*, 157:915-920 (2002)), using well-defined criteria (McLellan et al., *Carcinogenesis*, 12:2093-8 (1991). Mice were analyzed at 40 weeks for tumor number, size, location, and pathological grade as described elsewhere (Gokmen-Polar et al., *Cancer Res.*, 61:1375-81 (2001)). All tumors were classified as either tubular adenomas or intramucosal carcinomas (carcinoma in situ) by a board-certified pathologist. Digital images of the tumors were captured using a Nikon Eclipse E600 microscope equipped with a ProgRes C14 camera (Jenoptik, Jena, Germany) using a 20× objective lens. Images were acquired using ProgRes C14 software with Microsoft Photoeditor and processed with Microsoft Photoshop.

Transgenic K-ras^{LA2} mice (Johnson et al., *Nature*, 410:1111-6 (2001); provided by T. Jacks, M.I.T., Boston, Mass.) were bred to transgenic kdPKC$_\iota$ mice to obtain bi-transgenic K-ras^{LA2}/kdPKC$_\iota$ mice. At 12 weeks of age, transgenic K-ras^{LA2} and transgenic K-ras^{LA2}/kdPKC$_\iota$ mice were assessed for spontaneous ACF formation (McLellan et al., *Carcinogenesis*, 12:2093-8 (1991) and Murray et al., *J. Cell Biol.*, 145:699-711 (1999)).

RIE Cell Transfections and Cellular Analyses

[0066] RIE cells and derivatives were grown in DMEM containing 5% FBS as described elsewhere (Ko et al., *Oncogene*, 16:3445-54 (1998)). RIE/Ras cells were described elsewhere (Sheng et al., *J. Biol. Chem.*, 275:6628-35 (2000); provided by Dr. H. M. Sheng, University of Texas Medical Branch, Galveston, Tex.). Microarray analysis of RIE/Ras cells demonstrated that these cells express no PKCζ. cDNAs encoding human wtPKC$_\iota$ and kdPKC$_\iota$ were cloned into the pBABE/FLAG/puro retroviral expression vector, and virus stocks were produced using Phoenix-E cells (provided by Dr. G. Nolan, Stanford University, Palo Alto, Calif.). Puromycin-

resistant, stable transfectants were generated. Expression of FLAG-epitope-tagged PKC_ι was confirmed by immunoblot analysis using an anti-FLAG antibody (Sigma-Aldrich), and PKC_ι kinase activity was determined by immunoprecipitation histone kinase assay as described elsewhere (Jamieson et al., *J. Biol. Chem.*, 274:3927-3930 (1999)).

[0067] Recombinant retroviruses containing a dominant negative Rac1 (RacN17) that is Myc-tagged or a Myc-tagged RacV12 were generated by excising the Myc-tagged Rac1 constructs from pEXV/Rac vectors (Qiu et al., *Nature*, 374: 457-9 (1995)) with EcoRI and ligating them into the EcoRI site of the LZRS-GFP retrovirus. The entire coding sequence of each construct was confirmed by DNA sequence analysis. LZRS-GFP-Rac1 retroviruses were used to infect RIE cells and derivative cell lines as described elsewhere (Ireton et al., *J. Cell Biol.*, 159:465-76 (2002)). Rac1 activity was assessed by affinity-isolation of GTP-bound Rac1 as described elsewhere (Sander et al., *J. Cell Biol.*, 143:1385-98 (1998)). Active GTP-bound Rac1 and total Rac1 were identified by immunoblot analysis using a Rac1 monoclonal antibody (BD Transduction Laboratories) and quantitated by densitometry.

[0068] Invasiveness of RIE cell transfectants was assessed in Transwell inserts pre-coated with Matrigel (6.5 mm diameter, 8 μm pore size; BD Biosciences). DMEM containing 10% FBS was added to the lower chamber and 5×10^4 cells were suspended in serum-free DMEM (500 μl) and placed in the top chamber of the Transwell insert. Cells were incubated for 22 h at 37° C. in 5% CO_2, at which time non-invading cells were removed from the upper chamber. Cells that had invaded through the Matrigel-coated filter were fixed in 100% methanol, stained with Crystal Violet and counted on a microscope using a calibrated ocular grid. Fifteen representative areas of the lower chamber were counted to determine the number of invasive cells in each well.

To assess anchorage-independent growth, RIE cell transfectants were suspended in DMEM supplemented with 10% FBS, 1.5% agarose, and a 1% insulin, transferrin and selenium solution (Sigma-Aldrich), and plated (300 cells/60 mm dish) on a layer of 1.5% agar containing the same medium. Cell colonies were fixed with 20% methanol and stained with Giemsa after 7-14 days in culture and quantified under a dissecting microscope.

Example 2

PKC_ι Expression

[0069] The potential involvement of PKC_ι in colon carcinogenesis was assessed by determining expression of PKC_ι in normal mouse colonic epithelium and in colon tumors induced by the carcinogen, azoxymethane (AOM). Immunoblot analysis demonstrated that PKC_ι expression is elevated in AOM-induced colon tumors when compared to matched, uninvolved colonic epithelium (FIG. 1a). Reverse transcriptase-(RT)-PCR analysis demonstrated a corresponding increase in PKC_ι mRNA in these tumors (FIG. 1b). Immunoblot analysis demonstrated that PKC_ι expression is also elevated in human colon carcinoma specimens when compared to matched uninvolved colonic epithelium (FIG. 1c), demonstrating that elevated PKC_ι is a common feature of AOM-induced mouse colon tumors and human colon carcinomas.

[0070] Immunohistochemical staining confirmed the elevated expression of PKC_ι in AOM-induced colon tumors in mice when compared to normal adjacent colonic epithe-

lium (FIG. 2). Significant expression of PKC_ι was detected in normal colonic epithelium (FIG. 2, panel a), but much stronger staining was observed in colon tumor tissue (FIG. 2, panel b), consistent with our immunoblot analysis. Specificity of the immunostaining was assessed by staining sections from the same tissue with an antibody dilution to which had been added a five-fold molar excess of a peptide corresponding to the epitope on PKC_ι used to generate the PKC_ι antibody (FIG. 2, panels c and d). Inclusion of the blocking PKC_ι peptide abolished the immunostaining, confirming the specificity for PKC_ι.

Example 3

Carcinogenesis

[0071] The elevated expression of PKC_ι in colon tumors indicated that PKC_ι may play an important role in colon carcinogenesis. To test this hypothesis, transgenic mice were generated to express either a constitutively active (caPKC$_\iota$) or kinase-deficient (kdPKC$_\iota$) form of human PKC_ι in the colonic epithelium using a modified rat liver fatty acid binding protein promoter (Murray et al., *J. Cell Biol.*, 157:915-920 (2002) and Simon et al., *J. Biol. Chem.*, 272:10652-63 (1997)). Briefly, nucleic acid encoding caPKC$_\iota$ was generated by PCR-mediated site-directed mutagenesis and amplification of a fragment containing an alanine to glutamine (A^{120}E) substitution within the pseudosubstrate domain of human PKC_ι (FIG. 6). Nucleic acid encoding kdPKC$_\iota$ was created using a two-step PCR mutagenesis method to introduce a (K^{274}W) substitution at the ATP binding site of human PKC_ι (FIG. 7). Transgene constructs consisting of the Fabp1$^{4x\ at}_{-132}$ promoter (Simon et al., *J. Biol. Chem.*, 272:10652-10663 (1997)), the caPKC$_\iota$ or kdPKC$_\iota$ cDNA, and the SV40 large T antigen polyadenylation site were produced by conventional cloning techniques, and the sequences confirmed by direct microsequencing. The transgene constructs were propagated in the mammalian expression vector pREP4. The transgene inserts were excised from the cloning vector using NheI (5') and XbaI (3'), purified, and microinjected into C57BL/6J mouse oocytes as described elsewhere (Hogan et al., (1994) Manipulating the Mouse Embryo: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Press, Cold Spring Harbor, N.Y.). The microinjections and generation of transgenic founder mice were conducted at a transgenic mouse facility.

[0072] Immunoblot analysis demonstrated that transgenic caPKC$_\iota$ and kdPKC$_\iota$ mice express elevated PKC_ι protein in the colonic epithelium (FIGS. 3a and b, upper panels). Transgenic caPKC$_\iota$ mice exhibited high intrinsic PKC_ι activity in the colonic epithelium when compared to non-transgenic littermates (FIG. 3a, lower panel). In contrast, transgenic kdPKC$_\iota$ mice exhibited decreased colonic PKC_ι kinase activity when compared to non-transgenic littermates (FIG. 3b, lower panel; the autoradiograph in FIG. 3b is a longer exposure than FIG. 3a in order to reveal the decreased PKC_ι activity in transgenic kdPKC$_\iota$ mice). Neither transgenic caPKC$_\iota$ nor transgenic kdPKC$_\iota$ mice exhibited demonstrable changes in proliferative index, proliferation zone, or expression of differentiation markers in the colonic epithelium.

[0073] To assess the importance of PKC_ι in colon carcinogenesis, transgenic caPKC$_\iota$, transgenic kdPKC$_\iota$, and non-transgenic mice were treated with AOM (Gokmen-Polar et al., Cancer Res., 61:1375-81 (2001) and Murray et al., *J. Cell Biol.*, 145:699-711 (1999)). Initially, mice were analyzed 12 weeks after AOM treatment for the development of preneo-

9

plastic colonic lesions, aberrant crypt foci (ACF) (FIG. 3c). Heterozygous transgenic caPKC$_\iota$ mice developed about twice as many ACF, and homozygous caPKC$_\iota$ mice about three times as many ACF, as non-transgenic littermates (FIG. 3c). In contrast, homozygous transgenic kdPKC$_\iota$ mice developed significantly fewer ACF than non-transgenic mice. Thus, PKC$_\iota$ activity in the colonic epithelium correlates directly with susceptibility to AOM-induced ACF formation. ACF occur in both humans and mice and are considered to be precursors to colon tumors (McLellan et al., *Cancer Res.,* 51:5270-4 (1991) and Takayama et al., *N. Engl. J. Med.,* 339:1277-84 (1998)). ACF contain many of the same genetic and biochemical alterations found in colon tumors, including increased expression of PKCβII (Gokmen-Polar et al., *Cancer Res.,* 61:1375-81 (2001)) and activating K-Ras mutations (Shivapurkar et al., *Cancer Lett.,* 115:39-46 (1997)). Both the number and multiplicity (number of crypts/focus) of ACF are highly predictive of subsequent colon tumor formation in rodents (Magnuson et al., *Cancer Res.,* 53:4499-504 (1993)).

[0074] The effect of transgenic caPKC$_\iota$ expression on colon tumor formation was assessed. Transgenic caPKC$_\iota$ mice exhibited a three-fold higher incidence of tumors than non-transgenic control mice [63.6% (7/11) versus 20% (2/10) tumor-bearing mice]. In addition to an increase in tumor incidence, transgenic caPKC$_\iota$ mice developed predominantly malignant intramucosal carcinomas (6/7 tumors; FIG. 3e), whereas non-transgenic control mice developed mainly benign tubular adenomas (2/3 tumors; FIG. 3d). These results demonstrate that elevated PKC$_\iota$ activity in the colonic epithelium has two major effects on colon carcinogenesis. The first effect is an increase in formation of preneoplastic lesions and subsequent colon tumors. The second effect is to promote tumor progression from benign adenoma to malignant intramucosal carcinoma. Due to the low tumor incidence in non-transgenic mice it was impractical to assess whether transgenic kdPKC$_\iota$ mice would develop significantly fewer tumors.

Example 4

Ras Signaling

[0075] A relationship may exist between PKC$_\iota$ and Ras signaling (Coghlan et al., *Mol. Cell. Biol.,* 20:2880-9 (2000); Kampfer et al, *J. Biol. Chem.,* 276:42834-42 (2001); and Uberall et al., *J. Cell Biol.,* 144:413-25 (1999)). The importance of PKC$_\iota$ in Ras-mediated transformation of the intestinal epithelium was assessed. Rat intestinal epithelial (RIE) cells were used to study Ras-mediated transformation and to elucidate the molecular mechanisms by which PKCβII promotes a pro-carcinogenic phenotype (Murray et al., *J. Cell Biol.,* 157:915-920 (2002); Sheng et al., *J. Biol. Chem.,* 275: 6628-35 (2000); and Yu et al, *J. Biol. Chem.,* 278:11167-74 (2003)). RIE cells stably transfected with oncogenic V12H-ras (RIE/Ras) were transfected with FLAG-tagged-, wild-type (wt) PKC$_\iota$, or kdPKC$_\iota$. Both RIE/Ras/wtPKC$_\iota$ and RIE/Ras/kdPKC$_\iota$ cells expressed elevated levels of PKC$_\iota$ when compared to RIE or RIE/Ras cells (FIG. 4a, top panel). Immunoblot analysis using an antibody to oncogenic V12 Ras demonstrated that RIE/Ras, RIE/Ras/wtPKC$_\iota$, and RIE/Ras/kdPKC$_\iota$ cells express comparable levels of active oncogenic Ras (FIG. 4a, second panel). Actin immunoblot analysis confirmed that equal amounts of protein were loaded for each cell line (FIG. 4a, third panel).

[0076] Immunoprecipitation kinase assays (Jamieson et al., *J. Biol. Chem.,* 274:3927-3930 (1999)) were performed on RIE, RIE/Ras, RIE/Ras/wtPKC$_\iota$, and RIE/Ras/kdPKC$_\iota$ cells to assess total PKC$_\iota$ activity in these cell lines (FIG. 4a, fourth and fifth panels). Whereas RIE and RIE/Ras cells expressed equivalent levels of endogenous PKC$_\iota$ (FIG. 4a, fourth panel), RIE/Ras cells exhibited elevated PKC$_\iota$ activity as a result of the expression of oncogenic Ras (FIG. 4a, fifth panel). Thus, expression of oncogenic Ras leads to activation of endogenous PKC$_\iota$, while having no demonstrable effect on PKC$_\iota$ expression. RIE/Ras/wtPKC$_\iota$ cells expressed elevated levels of both PKC$_\iota$ protein and activity when compared to RIE or RIE/Ras cells, whereas RIE/Ras/kdPKC$_\iota$ exhibited elevated expression of PKC$_\iota$, but showed no increase in PKC$_\iota$ activity when compared to RIE/Ras cells (FIG. 4a, fourth and fifth panel). Immunoprecipitation with an anti-FLAG antibody followed by immunoblot analysis for PKC$_\iota$ confirmed the expression of FLAG-wtPKC$_\iota$ and FLAG-kdPKC$_\iota$ in RIE/Ras/wtPKC$_\iota$ and RIE/Ras/kdPKC$_\iota$ cells, respectively (FIG. 4a, sixth panel). PKC$_\iota$ kinase assay of anti-FLAG immunoprecipitates demonstrated that RIE/Ras/wtPKC$_\iota$ cells contain catalytically active, FLAG-wtPKC$_\iota$, whereas RIE/Ras/kdPKC$_\iota$ cells contain catalytically inactive FLAG-kdPKC$_\iota$ (FIG. 4a, seventh panel). Taken together, these data demonstrate that oncogenic Ras can activate both endogenous and transfected PKC$_\iota$, and confirm that the kdPKC$_\iota$ construct is deficient in kinase activity.

[0077] RIE/Ras cells exhibit an increase in anchorage-dependent growth rate and saturation density when compared to RIE cells (FIG. 4b). Expression of either wtPKC$_\iota$ or kdPKC$_\iota$ had little effect on the Ras-mediated increase in anchorage-dependent growth rate or saturation density (FIG. 4b). RIE cells expressing either wtPKC$_\iota$ or kdPKC$_\iota$ in the absence of oncogenic Ras exhibited no demonstrable change in growth rate compared to RIE cells and no signs of cellular transformation.

[0078] Ras transformation is dependent upon Ras-mediated activation of the small molecular weight GTPase, Rac1 (Qiu et al., *Nature,* 374:457-9 (1995)). Therefore, Rac1 activity in RIE/Ras cells was measured (FIG. 4c and d). RIE/Ras cells exhibit elevated Rac1 activity when compared to RIE cells (FIG. 4c). Expression of either a dominant negative Rac1 mutant, RacN17 (Qiu et al., *Nature,* 374:457-9 (1995)), or kdPKC$_\iota$ in RIE/Ras cells blocked Ras-mediated Rac1 activation. In contrast, expression of a constitutively active Rac1 mutant, RacV12 (Qiu et al., *Nature,* 374:457-9 (1995)), had little effect on Ras-mediated activation of endogenous Rac1. Expression of wild-type PKC$_\iota$ in the absence of oncogenic Ras was not sufficient to induce Rac1 activity (unpublished data). Thus, oncogenic Ras activates Rac1 in a PKC$_\iota$-dependent fashion.

[0079] Both Ras and Rac1 have been implicated in cellular motility and invasion (De Corte et al., *Embo. J.,* 21:6781-90 (2002)) and RIE/Ras cells exhibit an invasive phenotype (Fujimoto et al., *Exp. Cell Res.,* 266:239-49 (2001)). The following was used to assess whether the invasive phenotype observed in RIE/Ras cells is dependent upon Rac1 and PKC$_\iota$. RIE/Ras cells exhibited a highly invasive phenotype in Matrigel chambers, whereas RIE cells did not (FIG. 4d). Expression of either RacN17 or kdPKC$_\iota$ in RIE/Ras cells blocked Ras-mediated cellular invasion (FIG. 4d). However, expression of RacV12 in RIE/Ras/kdPKC$_\iota$ cells partially restored invasiveness. These results demonstrate that oncogenic Ras-mediated cellular invasion is dependent upon both Rac1 and

PKC_t. Interestingly, expression of either wild-type or constitutively active PKC_t in the absence of oncogenic Ras failed to induce invasion, indicating that PKC_t is necessary for oncogenic Ras-mediated invasion, but is not sufficient to induce invasion in the absence of oncogenic Ras.

[0080] RIE/Ras cells exhibited anchorage-independent growth in soft agar, whereas RIE cells did not (FIGS. 5a and b). Expression of $wtPKC_t$ significantly enhanced soft agar colony formation, while expression of $kdPKC_t$ blocked soft agar colony formation of RIE/Ras cells (FIGS. 5a and b). Furthermore, expression of RacV12 in RIE/Ras/$kdPKC_t$ cells restored the ability to form colonies in soft agar (FIG. 5c). Expression of RacV12 in RIE cells in the absence of oncogenic Ras did not induce soft agar colony formation, indicating that expression of active Rac1 is not sufficient to cause cellular transformation (FIG. 5c), consistent with previous reports that constitutively active Rac1 exhibits very weak transforming potential (Khosravi-Far et al., *Mol. Cell. Biol.,* 15:6443-53 (1995)). These data demonstrate that PKC_t plays a critical role in Ras-mediated transformation of RIE cells since PKC_t is required for Ras-mediated activation of Rac1, cellular invasion, and anchorage-independent growth. These results place PKC_t downstream of oncogenic Ras and upstream of Rac1 in a pathway that stimulates invasiveness and soft agar colony formation, two hallmarks of the transformed phenotype.

[0081] The importance of PKC_t in Ras-mediated colon carcinogenesis in vivo was assessed. For this purpose, a mouse model of Ras transformation consisting of a latent oncogenic K-ras allele (G12D) that is activated by spontaneous recombination in vivo was used (Johnson et al., *Nature,* 410:1111-6 (2001)). Latent K-ras (K-Ras^{LA2}) mice develop Ras-dependent lung carcinomas and ACF in the colonic epithelium (Johnson et al., *Nature,* 410:1111-6 (2001)). The transgenic $kdPKC_t$ mice were bred with K-Ras^{LA2} mice to generate bitransgenic K-Ras^{LA2}/$kdPKC_t$ mice, which were then assessed for spontaneous ACF development (FIG. 5d). K-Ras^{LA2}/$kdPKC_t$ mice developed significantly fewer ACF in the proximal colon than K-Ras^{LA2} mice. These data are consistent with the results in RIE/Ras cells in vitro, and demonstrate that PKC_t is critical for oncogenic K-ras-mediated colon carcinogenesis in vivo.

[0082] Taken together, these results provide direct evidence that PKC_t and Rac1 are necessary for the transformed phenotype induced by oncogenic Ras. Rac has previously been shown to be required for transformation by both H-Ras and K-Ras, the two most commonly mutated forms of Ras in human cancers. The data provided herein demonstrate that like, Rac1, PKC_t is also required for both H-Ras and K-Ras-mediated transformation. Whereas H-Ras and K-Ras have been shown to have both common and distinct effectors, recent evidence indicates that both of these Ras isoforms activate Rac 1, though K-Ras appears to be able to activate Rac1 more effectively than does H-Ras (Walsh et al., *J. Biol. Chem.,* 276:15609-15 (2001)). The data provided herein also demonstrate that H-Ras induces Rac1 activity through a PKC_t-dependent pathway and that PKC_t is required for K-Ras mediated colon carcinogenesis. Given the increased propensity of K-Ras to activate Rac 1, it is therefore quite likely that the Ras, PKC_t, Rac 1 pathway present in RIE cells is also involved in K-Ras-mediated colon carcinogenesis in vivo. Interestingly, PKC_t and Rac1 have also been implicated in the establishment of epithelial cell polarity through the formation of complexes containing PKC_t, the Par6 polarity

protein and Rac1 (Noda et al., *Genes Cells,* 6:107-19 (2001)). Rac1 is thought to regulate PKC_t activity within these complexes to affect cell polarity (Noda et al., *Genes Cells,* 6:107-19 (2001)). The data further implicate signaling through PKC_t/Par6/Rac1 complexes in Ras-mediated transformation.

[0083] These results provide conclusive evidence that PKC_t activity is critical for colonic epithelial cell transformation in vivo. However, disruption of PKC_t signaling (by expression of $kdPKC_t$) has little effect on normal intestinal epithelial cell homeostasis in vitro and in vivo. Taken together, these characteristics indicate that PKC_t can be an attractive target for development of novel therapeutics against colon cancer.

Example 5

PKC_t Expression Levels in Human Cancers

[0084] Immunoblot analysis and/or immunohistochemistry was used to examine the expression of PKC_t polypeptides in samples of human cancers and human cancer cell lines. Elevated expression of PKC_t polypeptides was detected in the following cancers: colon, lung, head and neck, ovary, esophagus, prostate, ovary, kidney, and pancreas. More than 80 patient cases of adenocarcinoma and squamous carcinoma of the lung for PKC_t expression were analyzed by both immunoblot analysis and immunohistochemistry using tissue arrays. Representative results are shown in FIG. 8. Without exception, these samples exhibited elevated PKC_t expression when compared to patient matched normal lung tissue. These results indicate that elevated expression of PKC_t can be a common feature of most, if not all, cancers.

Example 6

PKC_t and Lung Cancer

[0085] Non-small cell lung cancer (NSCLC) is the most common cause of cancer death in the United States. Long-term survival in NSCLC is low, indicating a need for better prognostic and therapeutic tools to detect and treat this disease. PKC_t is highly expressed in human non-small cell lung cancer cell lines and primary tumors, and is required for transformed growth of lung cancer cells in vitro and tumorigenicity in vivo. PKC_t activates a Rac1→Pak1→Mek1, 2→Erk1,2 signaling pathway that regulates lung cancer growth. In addition, the PKC_t gene is frequently amplified in lung squamous cell carcinoma cell lines and primary tumors, and PKC_t expression predicts poor survival in patients with lung adenocarcinoma.

Methods and Materials

[0086] Experimental Procedures Reagents: Antibodies were from the following sources and were used at the indicated concentrations: anti-PKCζ (Santa Cruz #sc-17640; 1:100), PKC_t (Transduction Labs #P20520; 1:4000), actin (Santa Cruz #sc-1616; 1:2000), the FLAG epitope (Sigma # A8592; 1:2000), Rac1 (Transduction Labs #610651; 1:3000), cIAP2 (Santa Cruz #sc-7944; 1:500), Bcl-XL (Cell Signaling #2762; 1:1000), PARP/cleaved PARP (Cell Signaling #9542; 1:1000), MEK, Phospho-(Ser217/221)-MEK and Phospho-(Ser298)-MEK (Cell Signaling #9122,9121 and 9128; 1:1000), ERK and Phospho-(Thr202/Tyr204)-ERK (Cell Signaling #9102/9101; 1:1000), CD31 (or Pecam-1; Santa Cruz #sc-1506; 1:1000), and BrdU (DAKO # M0744; 1:100). TUNEL staining was performed using the TdT-FragEL DNA

fragmentation detection kit (Calbiochem #QIA33). Recombinant human PKC_ι and $PKC\zeta$ polypeptides were obtained from Upstate Biochemical (#14-505 and #14-525, respectively). The myristoylated atypical PKC pseudosubstrate inhibitor peptide was obtained from Biosource (#77-749).

[0087] Cell Culture, Plasmids, Transfections and Drug Treatments: Human A549, ChaGo-K-1, H292, H520, H1299, and SK-MES-1 non-small cell lung cancer cell lines as well as the non-transformed HBE4 lung epithelial cell line were obtained from ATCC (Manassas, Va., USA) and maintained as suggested by the supplier. The cells were maintained in a humidified tissue culture incubator at 37° C. in 5% CO_2. A549, H1299, and ChaGo-K-1 cells were stably transfected with recombinant pBabe retroviruses containing Flag-tagged human full-length wild-type PKC_ι ($wtPKC_\iota$), kinase dead PKC_ι ($kdPKC_\iota$), or empty vector as described previously (Lu et al., *Oncogene*, 20:4777-4792 (2001)). Expression of FLAGepitope-tagged PKC_ι and total PKC_ι was analyzed by immunoblot analysis as described previously (Murray et al., *J. Cell Biol.*, 164:797-802 (2004)).

[0088] Adherent growth kinetics of A549 and H1299 cells transfected with empty pBabe, pbabe/$kdPKC_\iota$, or pbabe/wt-PKC_ι were determined by plating cells (1×10^4 cells/well) into multi-well culture dishes and monitoring cell growth daily over a seven day period. Each day, cells from triplicate wells were trypsinized and counted using a hemocytometer. In some experiments, A549 cells were maintained in medium containing either 10%, 2%, or no fetal bovine serum.

[0089] Cell Invasion and Soft Agar Growth Assays: A549 and H1299 transfectants were assayed for cell invasion using Matrigel-coated Transwell cell culture chambers (6.5-mm diameter, 8-μm pore size; BD Biosciences). A549 and H1299 cell transfectants in logarithmic growth phase were harvested with trypsin, the trypsin neutralized with serum-containing medium, and the cells pelleted and resuspended in serum-free growth medium. 2.5×10^4 cells were placed into the upper chamber of the Transwell insert, and growth medium containing 10% FBS was added to the lower chamber. After 22 hours at 37° C. in 5% CO_2, non-invasive cells in the upper chambers were removed and invasive cells were fixed in 100% methanol and stained with 0.5% crystal violet (Sigma) in 2% ethanol. Cells which had invaded through the Matrigel-coated filter were counted on a microscope (X40, Olympus) using a calibrated ocular grid.

[0090] Anchorage-independent growth was assayed by the ability of cells to form colonies in soft agar. The bottom agar consisted of growth medium containing 10% FBS and 0.75% agarose in 60-mm tissue culture dishes. Nine hundred cells were resuspended in growth medium containing 10% FBS and 0.75% agarose, and plated on top of the bottom agar. The cells were incubated at 37° C. in 5% CO_2. Cell colonies were visualized and quantified under a dissecting microscope (Olympus) after 4-6 weeks in culture.

[0091] Rac 1 Activity Assays: Rac1 activity in A549 and H1299 cell transfectants was assessed by affinity isolation of GTP-bound Rac1 using binding domains of PAK as described previously (Sander et al., *J. Cell Biol.*, 143(5):1385-98 (1998)). Briefly, cells were lysed in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 20 mM $MgCl_2$, 5 mM EGTA, 10% glycerol, 1% Triton X-100, 1% NP-40, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin) at 4° C. for 5 min. Cellular debris was removed by centrifugation at 20,000×g for 5 min, and supernatants were transferred to new

tubes containing 20 μl of GST-p21-binding domain of PAK1 (PAK1-PBD) coupled to agarose beads (Upstate). An aliquot of each supernatant was reserved to determine total Rac1 and actin expression by immunoblot analysis. Following a 30-minute incubation at 4° C., the agarose beads were collected by centrifugation and washed three times in wash buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 20 mM $MgCl_2$, 5 mM EGTA, 10% glycerol, 1% Triton X-100, 1% NP-40, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Bound polypeptides were solubilized by the addition of 30 μl of SDS sample buffer, resolved by SDS-PAGE, and subjected to immunoblot analysis for Rac1. An equivalent aliquot of the total cell lysate was subjected to immunoblot analysis to determine total Rac1 expression.

[0092] NF-κB Transcriptional Activity Assays: NF-κB transcriptional activity was assayed using a dual-luciferase reporter system (Promega) as described previously (Lu et al., *Oncogene*, 20:4777-4792 (2001)). In brief, A549 cells stably expressing $kdPKC_\iota$ or pBabe vector control were transiently transfected with 500 ng of 3×MHCLuc, a plasmid containing three NF-κB response elements from the MHC promoter linked to a luciferase reporter gene, and 25 ng of phRL-SV40 using the FuGene6 lipofection reagent (Roche Applied Science) as described by the manufacturer. Twenty four hours after transfection, NF-κB activity was stimulated with 50 ng/ml TNFα (R&D Systems) for 2 hours. Total cell extracts were prepared for the dual-luciferase assay according to manufacturer's (Promega) instructions. Firefly and Renilla luciferase activity were measured using a Veritas Microplate Luminometer (Turner BioSystems). The activity of Renilla luciferase was used as an internal control for transfection efficiency.

[0093] Tumorigenicity in Nude Mice: The growth of stably infected A549 human lung carcinoma cells as established subcutaneous tumors was studied in athymic nude mice (Harlan-Sprague-Dawley, Indianapolis, Ind.) in a defined pathogen-free environment. Briefly, A549 cell transfectants were grown in F-12K Nutrient Mixture containing 10% FBS. A549 cell transfectants were harvested and resuspended in serum-containing medium. 4-6 week old female nude mice were injected subcutaneously into the flank with 5×10^6 cells in 100 μl of growth medium. Once palpable tumors were established, tumor size was measured once a week. Tumor growth was quantified by measuring the tumors in three dimensions with calipers. Tumor volume (mm^3) was calculated using the formula: 0.5236 (L×W×H), where L represents the length of the tumor, W represents the width of the tumor, and H represents the height of the tumor. Animals were individually monitored throughout the experiment. At the conclusion of the study, mice were injected intraperitoneally with 100 μg/g of 5-bromo-2-deoxyuridine (BrdU) 1 hour prior to sacrificing the mice by CO_2 asphyxiation. Tumors were excised and divided into sections for protein extraction and tumor fixation. Total tumor extracts were prepared in SDS buffer [2% (w/v) SDS, 4 M urea, 62.5 mM Tris-HCl (pH 6.8), 1 mM EDTA, 5% (v/v) β-mercaptoethanol] and equal amounts of polypeptide were subjected to immunoblot analysis as described herein. A section of tumor was also fixed in 10% buffered formalin, embedded in paraffin, sectioned (5 μm thickness), and stained for appropriate antigens.

[0094] Immunoblot Analysis Cells were harvested by washing with PBS and scraping off the plate. The cell pellet was lysed in SDS sample buffer. Protein lysates were quan-

titated by using the nitration of tyrosine in nitric acid (Bible et al., *Anal. Biochem.*, 267(1):217-21 (1999)). Equal amounts of protein (~20 μg) were loaded for each sample, resolved in 12% or 4-20% SDS-PAGE gels (Invitrogen) and transferred to PVDF membrane (Millipore Immobilin-P). A solution of 5% milk and PBS-Tween 20 was used for blocking TBS-Tween 20 was used for phospho-specific antibodies. Western blot analysis was performed with appropriate antibodies and detected using ECL-Plus (Amersham).

[0095] Analysis of Human Lung Cancer Tissues: H&E stained sections of matched normal and lung tumor tissues were analyzed by a pathologist in order to confirm initial diagnosis, staging, and overall integrity of the tissue samples. Based on this analysis, 40 cases of squamous cell carcinoma of the lung, 40 cases of adenocarcinoma of the lung, and matched normal lung tissues were chosen for extraction of DNA, RNA, and protein. Ten 10 μm thick slices were cut from each frozen block. DNA was isolated in phenol/chloroform, total RNA was isolated using RNAqueous 4PCR kit (Ambion), and protein was isolated by direct solubilization in SDS-PAGE sample buffer.

[0096] Real Time PCR Analysis for PKC_ι Gene Amplification: Genomic DNA from each sample was analyzed for amplification of PKC_ι using TaqMan technology on an Applied Biosystems 7900HT sequence detection system. The human RNaseP1 gene was used as a DNA template control and for normalization of results to total DNA. The primer/probe set for the human PKC_ι gene was as follows: forward primer, 5'-GGC-TGCATTCTTGCTTTCAGA-3' (SEQ ID NO:9); reverse primer, 5'-CCAAAAATA-TGAAGCCCAG-TAATCA-3' (SEQ ID NO:10); and probe: 5'-CAATCTTAC-CTG-CTTTCT-3' (SEQ ID NO:11). The primer/probe set for the RNAseP1 gene was designed and provided by ABI Assay on Demand.

[0097] Real-time Reverse Transcriptase-PCR Analysis of PKC_ι mRNA Abundance: PKC_ι mRNA abundance was determined by real-time Reverse Transcriptase-PCR using TaqMan technology on Applied Biosystems 7900HT sequence detection system. Human glyceraldehyde-3-phosphate dehydrogenase was used as an endogenous control. Samples were subjected to RT-PCR in the absence of reverse transcriptase controlled for the presence of genomic DNA. The primer/probe set for human PKC_ι mRNA spans the exon 16/17 border and was as follows: forward primer, 5'-CGT-TCTTCCGAAATGTTGAT-TG-3' (SEQ ID NO:12); reverse primer, 5'-TCCCCAGAAATATTTGGTTTAAAGG-3' (SEQ ID NO:13); and probe, 5'-TTGCTCCATCATATCC-3' (SEQ ID NO:14).

[0098] Analysis of PKC_ι Polypeptide Expression: Polypeptides from human tumor samples was quantified using nitric acid mediated nitration of tyrosine (Bible et al., *Anal. Biochem.*, 267(1):217-21 (1999)). Equal amounts of polypeptide (~30 μg) from each sample was resolved in 12% SDS-PAGE gels (Invitrogen), transferred to PVDF membrane (Millipore Immobilin-P), and subjected to immunoblot analysis using the appropriate antibodies and ECL-Plus detection (Amersham) as described previously (Murray et al., *J. Cell Biol.*, 164:797-802 (2004) and Zhang et al., *J. Biol. Chem.*, 279, 22118-22123 (2004)). Images were obtained on X-omat AR film, and antigens quantified by fluorescence detection using a Typhoon 9410 Variable Mode Imager. The fluorescent signal was analyzed using ImageQuant 5.2 software (Amersham).

[0099] Immunohistochemistry was performed on paraffin embedded sections of primary tumor and normal lung tissues. The tissue was deparaffinized by placing slides into 3 changes of xylene and rehydrated in a graded ethanol series. The rehydrated tissue samples were rinsed in water and subjected to antigen retrieval in citrate buffer pH 6.0 as described by the manufacturer (Dako). Slides were treated with 3% H_2O_2 for five minutes to reduce endogenous peroxidase activity and washed with PBS containing 0.5% (w/v) Tween 20. PKC_ι was detected using PKC_ι antibody at a 1:100 dilution in PBS/Tween and visualized using the Envision Plus Dual Labeled Polymer Kit following the manufacturer's instructions (Dako). Images were captured and analyzed using ImagePro software.

[0100] Statistical and Survival Analysis: Cancer-specific survival was estimated using the Kaplan-Meier method. The duration of follow-up was calculated from the sample date to the date of death or last follow-up. The associations of the clinical and pathologic features studied with death from lung cancer were assessed using Cox proportional hazards regression models and summarized with risk ratios and 95% confidence intervals (CI). Natural logarithmic transformations were explored if the distributions of continuously scaled variables were not approximately normal. In addition, the relationships between continuously scaled variables and death from lung cancer were investigated using martingale residuals from the Cox model (Therneau et al., Modeling Survival Data: Extending the Cox Model. First edition Ann Arbor, Springer-Verlag, (2000)). Statistical analyses were performed using the SAS software package (SAS Institute; Cary, N.C.) and p-values <0.05 were considered statistically significant.

Results

[0101] Atypical PKC_ι Expression is Elevated in Human NSCLC Cells: The expression of PKC_ι and PKCζ in established human NSCLC cell lines was assessed. Immunoblot analysis of total cell lysates from six human NSCLC cell lines (A549, H1299, H292, ChaGoK1, Sk-Mes1, and H520) revealed that each cell line expressed elevated levels of PKC_ι when compared to non-transformed human HBE4 lung epithelial cells (FIG. 10A). In contrast, none of the cell lines expressed detectable levels of PKCζ. Purified recombinant human PKC_ι and PKCζ were included in the immunoblot analyses to ensure the specificity and activity of the antibodies for their respective antigens. Quantitative real time PCR analysis of isolated RNA revealed an increase in PKC_ι mRNA abundance in each of the NSCLC cell lines compared to HBE4 cells. In contrast, PKCζ mRNA was detected at much lower levels in each cell line and was not elevated in NSCLC cell lines. Thus, PKC_ι is the major, and perhaps the only atypical PKC isozyme expressed in non-transformed lung epithelial and NSCLC cells, and PKC_ι expression is elevated in NSCLC cell lines when compared to non-transformed lung epithelial cells.

[0102] PKC_ι is Required for Human NSCLC Cell Transformation in vitro: Having identified PKC_ι as the major atypical PKC isozyme expressed in human NSCLC cell lines, the role of PKC_ι in the transformed phenotype exhibited by lung cancer cells was examined. A549 cells, a commonly studied LAC cell line, were stably transfected with retroviruses expressing either wild type human PKC_ι (wtPKC_ι), a kinase deficient PKC_ι mutant (kdPKC_ι) which acts in a dominant negative fashion (Jamieson et al., *J. Biol. Chem.*, 274, 3927-

3930 (1999) and Murray et al., *J. Cell Biol.*, 164:797-802 (2004)), or empty retroviral vector (pBabe). Immunoblot analysis using an anti-Flag antibody confirmed expression of the appropriate recombinant PKC$_t$ polypeptides and a PKC$_t$-specific antibody monitored total PKC$_t$ polypeptide expression (FIG. **10**B). No significant change in growth rate, saturation density, or survival was observed in any of the A549 cell transfectants grown in adherent culture in 10% serum, 2% (reduced) serum, or in the absence of serum (FIG. **10**C). Thus, PKC$_t$ signaling does not appear to be important for growth or survival of A549 cells in adherent culture.

[0103] Despite having no effect on adherent growth or survival of A549 cells, expression of kdPKC$_t$ had a dramatic inhibitory effect on several aspects of the transformed phenotype of A549 cells. Thus, A549/pBabe and A549/wtPKC$_t$ cells exhibited a highly invasive phenotype as measured by invasion through Matrigel-coated chambers, whereas A549/kdPKC$_t$ cells showed a significantly reduced invasive potential (FIG. **10**D). Similarly, both A549/pBabe and A549/wtP-KC$_t$ cells form abundant colonies in soft agar, whereas A549/kdPKC$_t$ cells exhibit a significant impairment in anchorage-independent growth (FIG. **10**E). Thus, PKC$_t$ appears to be involved in the transformed phenotype of A549 cells and cellular invasion and anchorage-independent growth.

[0104] To assess whether the effects of kdPKC$_t$ on transformation were specific to A549 cells, H1299 cells, a SCC cell line, stably expressing either wtPKC$_t$ or kdPKC$_t$ were established (FIG. **11**A). Consistent with the results in A549 cells, expression of either wtPKC$_t$ or kdPKC$_t$ had no effect on anchorage-dependent cell growth of H1299 cells (FIG. **11**B). However, kdPKC$_t$ significantly inhibited both cellular invasion (FIG. **11**C) and anchorage-independent growth of H1299 cells in soft agar (FIG. **11**D), whereas wtPKC$_t$ had little or no effect. Expression of kdPKC$_t$ in ChaGoK1 cells, another SCC cell line, resulted in a similar inhibition of transformed growth in soft agar (FIG. **11**E). Therefore, PKC$_t$ signaling is involved in the transformation of both SCC and LAC, and is not peculiar to A549 cells.

[0105] Rac1 is a Downstream Target of PKC$_t$ in Lung Cancer Cell Transformation: The relative importance of Rac1 and NF-κB in mediating PKC$_t$-dependent transformation of NSCLC cells was assessed (FIG. **12**). Both A549/pBabe and H1299/pBabe cells exhibited significant Rac1 activity, as assessed by the level of GTP-bound Rac1, which is inhibited by the expression of kdPKC$_t$ but not wtPKC$_t$ (FIG. **12**A). These results are consistent with the results from Ras-transformed RIE cells which exhibited a similar inhibition of Rac1 activity by kdPKC$_t$ expression (Murray et al., *J. Cell Biol.*, 164:797-802 (2004)).

[0106] The involvement of NF-κB signaling in PKC$_t$-dependent transformation was also assessed. NF-κB plays a role in the protection of NSCLC cells from apoptosis through direct transcriptional induction of expression of the antiapoptotic genes cIAP2 and Bcl-XL (Cheng et al., *Oncogene*, 19, 4936-4940 (2000); Jiang et al., *Oncogene*, 20, 2254-2263 (2001); and Webster et al., *Endocrinology*, 143, 3866-3874 (2002)). Neither wtPKC$_t$ nor kdPKC$_t$ had an effect on the steady-state levels of cIAP2 or Bcl-XL in either A549 or H1299 cells (FIG. **12**B). Furthermore, neither wtPKC$_t$ nor kdPKC$_t$ induced apoptosis in A549 or H1299 cells as measured by caspase-mediate cleavage of PARP (FIG. **12**C) or trypan blue exclusion viability analysis. Inhibition of NF-κB transcriptional activity in A549 and H1299 cells induces apoptosis (Jiang et al., *Oncogene*, 20, 2254-2263 (2001)). Direct

measurement of NF-κB transcriptional activity revealed that A549 cells exhibited significant basal and TNFα-stimulated NF-κB activity that is not affected by expression of kdPKC$_t$ (FIG. **12**D). Taken together, these results demonstrate that PKC$_t$ regulates Rac1 activity in A549 and H1299 cells. However, PKC$_t$ does not appear to be required for NF-κB signaling in these cells.

[0107] These results are interesting in light of the results obtained in other cell systems. For instance, in CML cells, PKC$_t$ is required for Bcr-Abl-mediated transformation and NF-κB was identified as a requisite downstream effector of PKC$_t$-dependent cell survival (Jamieson et al., *J. Biol. Chem.*, 274, 3927-3930 (1999); Lu et al., *Oncogene*, 20:4777-4792 (2001); and Murray et al., *J. Biol. Chem.*, 272, 27521-27524 (1997)). In contrast, in rat intestinal epithelial (RIE) cells, the small molecular weight GTPase Rac1 was identified as a downstream effector of oncogenic Ras-mediated, PKC$_t$-dependent transformation (Murray et al., *J. Cell Biol.*, 164:797-802 (2004)). Thus, it appears that PKC$_t$ can contribute to transformation through activation of at least two different signaling pathways depending upon the cellular context.

[0108] The PB1 Domain is Involved in PKC$_t$-dependent Transformation: The ability of kdPKC$_t$ to block Rac1 activity suggests that the kinase activity of PKC$_t$ is required for Rac1 activation in NSCLC cells. Treatment of A549 cells with the highly selective cell permeant atypical PKC pseudosubstrate peptide inhibitor, PSI, also blocks Rac1 activity (FIG. **12**E), confirming the involvement of PKC$_t$ activity in Rac1 activation. PKC$_t$ regulates Rac1 through PB1 domain-mediated complex formation between PKC$_t$, Rac1, and the adapter protein Par 6 (Etienne-Manneville et al., *Curr. Opin. Cell Biol.*, 15, 67-72 (2003)). It is possible that expression of the PB1 domain of PKC$_t$ would act as a competitive inhibitor of PKC$_t$-mediated activation of Rac1. Indeed, A549 cells stably transfected with a plasmid containing the first 113 amino acids of PKC$_t$, PKC$_t$ (1-113), which encompasses the PB1 domain of the PKC$_t$, inhibits Rac1 activity (FIG. **12**E). Furthermore, expression of PKC$_t$ (1-113) inhibits both A549 cell invasion (FIG. **12**F) and anchorage-independent growth in soft agar (FIG. **12**G), indicating the involvement of the PB1 domain in PKC$_t$-dependent activation of Rac1 and cellular transformation.

[0109] The PKC$_t$-Rac1 Signaling Axis is Required for Lung Cancer Cell Tumorigenicity in vivo: Since Rac1 was identified as a molecular target for PKC$_t$ in NSCLC cells, Rac1 was assessed for the ability to be a downstream effector of PKC$_t$-dependent transformation. Expression of a constitutively active mutant of Rac1, RacV12, restores transformed growth of A549/kdPKC$_t$ cells in soft agar (FIG. **13**A). Thus, Rac1 appears to be both necessary and sufficient for PKC$_t$-dependent transformation in vitro. The involvement of the PKC$_t$-Rac1 signaling axis in A549 cell tumorigenicity was assessed in vivo. Athymic nude mice were inoculated subcutaneously with A549/pBabe, A549/kdPKC$_t$, or A549/kdP-KC$_t$/RacV12 cells, and tumor growth was assessed over time. Expression of kdPKC$_t$ in A549 cells resulted in significant inhibition of tumor growth in vivo, whereas tumor growth was restored to levels indistinguishable from A549/pBabe cells by expression of RacV12 in A549/kdPKC$_t$ cells (FIG. **13**B). Taken together, these results demonstrate the involvement of the PKC$_t$-Rac1 signaling axis in A549 tumorigenicity in vivo.

[0110] The status of Rac1 and NF-κB signaling in tumors derived from A549 cell transfectants was also assessed. Rac1

14

activity in A549 cell tumors was measured by monitoring the level of activity of the downstream Rac1 effector MEK 1/2. MEK 1/2 was demonstrated to be a PKC_ι- and Rac1-dependent molecular target in Ras-transformed RIE cells (Murray et al., *J. Cell Biol.*, 164:797-802 (2004)). Immunoblot analysis of lysates from A549/pBabe cell tumors revealed significant levels of activated MEK that is phosphorylated on the Ser217/221 Raf activation sites on MEK1/2 (FIG. **13C**). Likewise, significant levels of active ERK, phosphorylated on the MEK-specific Thr202/Tyr204 phosphorylation sites, were detected in these tumors, indicating MEK/ERK activation (FIG. **13C**). In addition, significant phosphorylation was observed on the PAK1-specific phosphorylation site on MEK1/2, Ser298 in A549/pBabe tumors (FIG. **13C**). In contrast, A549/kdPKC$_\iota$ cell tumors exhibit reduced levels of phospho-MEK at both Raf- and Pak1-mediated sites with a concomitant decrease in phospho-ERK levels. A549/kdP-KC$_\iota$/RacV12 cell tumors exhibit phospho Ser217/221-MEK, phospho-Ser298 MEK, and phospho-Thr202/Tyr204-ERK levels indistinguishable from A549/pBabe cells. Taken together, these results demonstrate that PKC$_\iota$ regulates the MEK/ERK pathway in A549 cell tumors in vivo and indicate that a PKC$_\iota$/Rac1/PAK1/MEK/ERK pathway is involved in A549 cell tumorigenicity.

[0111] Though no evidence for PKC$_\iota$-dependent NF-κB activation in A549 cells was detected in vitro, it was possible that PKC$_\iota$ may be involved in maintenance of NF-κB activity and tumor survival in the in vivo setting. However, immunoblot analysis demonstrated that expression of the NF-κB transcriptional targets cIAP2 and Bcl-Xl were not affected by expression of kdPKC$_\iota$ or RacV12 (FIG. **13D**). In addition, no evidence for induction of apoptosis in tumors expressing kdPKC$_\iota$ or RacV12 was found as measured by caspase-mediated cleavage of PARP (FIG. **13D**). Likewise, Tunel analysis of A549/pBabe, A549/kdPKC$_\iota$, and A549/kdPKC$_\iota$/RacV12 cell tumors revealed very low levels of apoptosis in all tumors (apoptotic index of <0.2%) and no significant difference among the three tumor groups. Thus, it appears unlikely that NF-κB is a critical target for PKC$_\iota$-dependent tumorigenicity of A549 cells in vivo.

[0112] PKC$_\iota$ is Critical for Tumor Cell Proliferation in vivo: The mechanism by which kdPKC$_\iota$ inhibits A549 tumor formation in vivo was assessed. As described herein, induction of apoptosis in A549 cells expressing kdPKC$_\iota$ was not observed, indicating that kdPKC$_\iota$ does not inhibit tumor formation by impairing tumor cell survival. However, BrdU labeling of A549 cell tumors revealed that A549/kdPKC$_\iota$ tumors exhibited a significant decrease in BrdU-positive, cycling tumor cells when compared to A549/pBabe tumors (FIG. **14A**). In A549/kdPKC$_\iota$/RacV12 tumors, the BrdU labeling index is indistinguishable from that of A549/pBabe cell tumors. Quantitative measurement of BrdU-labeled cells revealed a 2.5-3-fold reduction in proliferative index in A549/kdPKC$_\iota$ tumors compared to A549/pBabe tumors that was completely restored by expression of RacV12 (FIG. **14B**). Thus, PKC$_\iota$ plays a role in A549 cell tumor proliferation.

[0113] It is possible that A549/kdPKC$_\iota$ tumors exhibit a reduced proliferative index due to a reduction in tumor vascularization as a result of decreased angiogenesis. However, immunohistochemical staining with the endothelial cell marker CD31 revealed no change in tumor-associated vessel density in A549 cell tumors in the presence of kdPKC$_\iota$ or RacV12 (FIG. **14C**). Immunoblot analysis confirmed that A549/pBabe, A549/kdPKC$_\iota$, and A549/kdPKC$_\iota$/RacV12

tumors contained similar levels of CD31 polypeptide (FIG. **14D**). Taken together, these results indicate that PKC$_\iota$ is necessary for A549 tumor cell growth by activating a Rac1, PAK1, MEK, ERK signaling pathway while having little or no effect on tumor cell survival or tumor vascularization.

[0114] PKC$_\iota$ Expression is Elevated in Primary Squamous Cell Carcinomas: The results provided herein demonstrate that PKC$_\iota$ expression is elevated in NSCLC cells, and that PKC$_\iota$ plays a role in NSCLC cell transformation in vitro and in vivo. In order to determine whether PKC$_\iota$ expression is relevant to human disease, atypical PKC expression in the two major sub-types of NSCLC, SCC and LAC, were assessed. Forty cases of SCC and matched normal lung tissues were initially selected for analysis. Three cases had received therapy prior to obtaining the tissue samples and were excluded from the analysis in order to eliminate the possible effect of treatment on PKC$_\iota$ expression. A fourth case was excluded because sufficient protein could not be obtained from both the normal and tumor tissues. The remaining 36 cases were analyzed by immunoblot analysis for expression of PKC$_\iota$, PKCζ, and actin. Results from five representative cases are shown in FIG. **15A**. Elevated PKC$_\iota$ expression was evident in 35/36 (97%) cases when compared to matched normal lung tissue. PKCζ was not detected in any of the tumor or normal lung tissue samples, indicating that PKC$_\iota$ is the predominant atypical PKC expressed in benign and malignant human lung tissue. Real time PCR analysis demonstrated that PKC$_\iota$ mRNA was routinely 10 fold more abundant than PKCζ mRNA in normal and malignant lung tissues, confirming the predominance of PKC$_\iota$ in the human lung.

[0115] Quantitative analysis of the immunoblot data demonstrated a statistically significant increase in PKC$_\iota$ expression in SCC compared to normal lung tissue (FIG. **15B**). Elevated PKC$_\iota$ was confirmed by immunohistochemistry of all 36 cases contained on tissue microarrays. Light staining for PKC$_\iota$ was observed in normal lung epithelium with intense staining in tumor cells (FIG. **15C**). Little or no staining of stromal elements associated with the tumors was observed. PKC$_\iota$ staining was consistent with localization of the enzyme to the cytoplasm, plasma membrane, and nucleus of both normal lung epithelial and tumor cells. No obvious changes in cellular distribution of PKC$_\iota$ were observed between the normal and lung cancer tissues.

[0116] Whether PKC$_\iota$ polypeptide expression correlates with PKC$_\iota$ mRNA abundance was assessed in SCC tumors. Total RNA was isolated from 21 SCCs and matched normal samples and assessed for PKC$_\iota$ mRNA abundance by quantitative real time PCR. Spearman rank order analysis demonstrated a positive correlation between PKC$_\iota$ mRNA abundance and PKC$_\iota$ polypeptide expression in SCC (FIG. **15D**).

[0117] PKC$_\iota$ Gene Amplification Regulates PKC$_\iota$ Expression in SCC Cell Lines and Primary SCC Tumors: Among the cytogenetic changes commonly found in lung SCCs, amplification of chromosome 3q26 is among the most frequent, occurring in about 40-50 percent of SCCs (Balsara et al., *Cancer Res.*, 57, 2116-2120 (1997) and Brass et al., *Cancer Res.*, 57, 2290-2294 (1997)). Multiple candidate oncogenes reside in the chromosome 3q26 region including the Ski-like gene SnoN (Imoto et al., *Biochem. Biophys. Res. Commun.*, 286, 559-565 (2001)), the catalytic subunit of phosphatidylinositol-3 kinase (PI3Kα) (Singh et al., *Genes Dev.*, 16, 984-993 (2002)), the Evil oncogene (Imoto et al., *Biochem. Biophys. Res. Commun.*, 286, 559-565 (2001)), and the RNA

15

component of human telomerase (TERC) (Yokoi et al., *Clin. Cancer Res.*, 9, 4705-4713 (2003)). However, the importance of these genes in SCC formation has not been systematically evaluated. Since the human PKC$_\iota$ gene resides at 3q26, whether PKC$_\iota$ gene amplification occurs in SCC cell lines and primary tumors was assessed. Quantitative real time PCR analysis revealed PKC$_\iota$ gene amplification in 3 of the 4 established human SCC cell lines tested. Specifically, amplification was detected in H520, H1299, and ChaGo cells, but not in Sk-Mes1 or nontransformed HBE4 lung epithelial cells (FIG. **16**A). The presence of PKC$_\iota$ gene amplification was consistent with the presence of chromosome 3q26 amplification reported for these cell lines (Yokoi et al., *Clin. Cancer Res.*, 9, 4705-4713 (2003)), indicating that PKC$_\iota$ is part of the 3q26 amplicon. Quantitative real-time reverse transcriptase PCR and immunoblot analysis revealed a positive correlation between PKC$_\iota$ gene copy number, PKC$_\iota$ mRNA abundance, and PKC$_\iota$ polypeptide expression in these cell lines (FIG. **16**A). Taken together, these results demonstrate that PKC$_\iota$ gene amplification occurs frequently in human SCC cell lines, that PKC$_\iota$ resides within the chromosome 3q26 amplicon, and that PKC$_\iota$ gene amplification is a mechanism by which PKC$_\iota$ expression is regulated in SCC cells.

[0118] Whether PKC$_\iota$ gene amplification occurs in primary SCC tumors was assessed. Genomic DNA isolated from 36 SCC cases was analyzed for PKC$_\iota$ gene copy number by quantitative real time PCR. Amplification was quantitated by normalizing PKC$_\iota$ gene copy number to the single copy RNAse P gene and standardized to patient-matched normal lung tissue. PKC$_\iota$ gene amplification was observed in 17/36 (47.2%) of the cases. Statistical analysis demonstrated a significant increase in PKC$_\iota$ gene copy number in SCC compared to matched normal lung tissue (FIG. **16**B). In addition, Spearman rank order analysis revealed a positive correlation between PKC$_\iota$ gene copy number and PKC$_\iota$ protein expression (FIG. **16**C), demonstrating that gene amplification is a mechanism by which PKC$_\iota$ expression is regulated in SCC tumors. This result is consistent with the reported 40-50% frequency of chromosome 3q26 amplification in SCCs (Balsara et al., Cancer Res., 57, 2116-2120 (1997) and Brass et al., *Cancer Res.*, 57, 2290-2294 (1997)). Taken together, these results demonstrate that PKC$_\iota$ expression is elevated in virtually all SCCs, that the PKC$_\iota$ gene is frequently amplified in these tumors, and that the PKC$_\iota$ gene resides within the previously described chromosome 3q26 amplicon. With the functional data showing the involvement of PKC$_\iota$ signaling in lung cancer cell growth and tumorigenicity, these results provide compelling evidence that PKC$_\iota$ is a relevant target for gene amplification with chromosome 3q26 that promotes squamous cell carcinogenesis. Chromosome 3q26 amplification also occurs frequently in SCC of the head and neck (Snaddon et al., *Br. J. Cancer*, 84, 1630-1634 (2001)), esophagus (Imoto et al., *Biochem. Biophys. Res. Commun.*, 286, 559-565 (2001) and Pimkhaokham et al., *Jpn. J. Cancer Res.*, 91, 1126-1133 (2000)), cervix (Sugita et al., *Cancer Genet. Cytogenet.*, 117, 9-18 (2000)) and ovary (Balsara et al., *Cancer Res.*, 57, 2116-2120 (1997) and Sonoda et al., *Genes Chromosomes Cancer*, 20, 320-328 (1997)). Therefore, the PKC$_\iota$ gene appears to be frequently amplified in these tumors as well.

[0119] PKC$_\iota$ Expression is Elevated in Lung Adenocarcinomas: Whether PKC$_\iota$ expression is elevated in LAC, the most prevalent form of NSCLC, was assessed. Forty primary LAC and matched normal lung tissue samples were initially

selected for analysis. Four cases received therapy prior to sample collection and were excluded from the analysis. Immunoblot analysis from five representative cases is shown in FIG. **17**A. As was observed with SCCs, PKC$_\iota$ was elevated in LACs when compared to matched normal lung tissue. PKCζ expression was not detected in either normal or cancerous lung tissue, indicating that just as in SCC, PKC$_\iota$ is the major atypical PKC isozyme expressed in LAC tumors.

[0120] The vast majority (33/36 or 91.7%) of LACs exhibited elevated PKC$_\iota$ expression, and Spearman rank order statistical analysis demonstrated a significant increase in PKC$_\iota$ polypeptide expression in LAC compared with matched normal lung tissue (FIG. **17**B). Immunohistochemical analysis of tissue microarrays made from LAC samples confirmed elevated PKC$_\iota$ staining in epithelial cells within the tumor with little or no immunostaining of surrounding stromal elements (FIG. **17**C). Real time PCR analysis revealed no PKC$_\iota$ gene amplification in any of the LAC samples, consistent with the rarity of chromosome 3q26 amplifications in LAC (Petersen et al., *Cancer Res.*, 57, 2331-2335 (1997)). Therefore, elevated PKC$_\iota$ expression is prevalent in both major forms of NSCLC.

[0121] PKC$_\iota$ Expression Predicts Poor Survival of Lung Adenocarcinoma Patients: The following was performed to determine whether PKC$_\iota$ polypeptide expression is of prognostic value for the assessment of patients with NSCLC. For this purpose, whether there is a correlation between PKC$_\iota$ expression and either disease stage or cancer-specific death in SCC and LAC was assessed. PKC$_\iota$ expression was determined on a continuous scale and normalized to matched normal lung tissue. In LAC, PKC$_\iota$ polypeptide expression correlated with an increased risk of cancer-specific death. Using Martingale residual analysis, the cases were divided into two groups based on PKC$_\iota$ expression. Patients in the high PKC$_\iota$ expression group were ten times more likely to die from LAC than patients in the low PKC$_\iota$ expression group (risk ratio 10.26, 95% CI 1.68-62.69; p=0.012) (FIG. **17**D). Cancer-specific death correlated positively with tumor stage since tumor stage is a reliable predictor of survival in LAC. In this cohort, patients with stage 3 tumors were 3.7 times more likely to die from LAC than were patients with stage 1 or stage 2 tumors (risk ratio 3.65; 95% CI 1.03-12.99; p=0.045). Interestingly, PKC$_\iota$ expression did not correlate with tumor stage, but rather is elevated to a similar degree in tumors at all stages. These data indicate that PKC$_\iota$ expression is an early event during lung carcinogenesis, and may be an important prognostic indicator of cancer-specific death in LAC patients independent of tumor stage. This finding has implications for the use of PKC$_\iota$ expression as a prognostic marker.

[0122] In SCC patients, a trend was observed between PKC$_\iota$ expression and cancer-specific death but the correlation did not reach statistical significance (p=0.21). The lack of a statistically significant correlation between PKC$_\iota$ expression and death in SCC may be due to molecular and genetic differences in these two forms of NSCLC. For instance amplification of the PKC$_\iota$ gene and other potential oncogenes present in the chromosome 3q26 amplicon in SCC may obscure a correlation between PKC$_\iota$ expression and clinical outcome. Alternatively, the small sample size analyzed may not have sufficient power to reveal a correlation between these parameters. Indeed, the well-established correlation between tumor grade and death from SCC, while observed in this patient data set, did not reach statistical significance (risk ratio 3.10, p=0.057), indicating that the sample size did not

provide sufficient power for the intended analysis. Additional analysis using a larger patient data set can be used to resolve between these possibilities. In conclusion, the results provided herein demonstrate that PKC_t plays a role in lung cancer cell transformation. The results that PKC_t is dispensable for adherent cell growth and survival indicate that PKC_t signaling is an attractive target for the development of new therapeutics for the treatment of lung cancer.

Example 7

Identifying Compounds that Inhibit PKC_t Binding, PKC_t Activity, and Tumorigenicity

[0123] A primary screen was performed as follows to identify test compound having the potential to inhibit the interaction of PKC_t polypeptides with PAR6 polypeptides. Bacterially expressed PKC_{t113}-YFP-N1 (PYN) was isolated from the soluble fraction of bacterial lysates by affinity purification according to the manufacturer's protocol (B-PER 6×His Purification Kit; Pierce). The purified polypeptide was dialyzed against Tris buffer (50 mM Tris, pH 8.0; 135 mM NaCl; 10% glycerol; 0.002% EDTA) containing 2 M urea. $PAR6_{125}$-CFP-C1 recombinant polypeptide was isolated from the inclusion body pellets of bacterial lysates using the B-PER reagent (Pierce) according to the manufacturer's protocol. The inclusion body pellet was solubilized in Tris buffer containing 8 M urea and dialyzed against the same buffer containing 4 M urea for 4 hours, and then overnight against Tris buffer containing 2 M urea. Yields were measured by fluorescence in a SpectraMax Gemini microplate reader (Molecular Devices).

[0124] To perform the assay, PYN was diluted to 4000 relative fluorescent units/50 μl in Tris buffer plus 1 M urea, and $PAR6_{125}$-CFP-C1 was diluted to 400 relative fluorescent units/50 μl Tris buffer plus 1 M urea. To each well of a 96-well, clear-bottom black plate (Costar 3631), 50 μl of PYN and 50 μl of $PAR6_{125}$-CFP-C1 were added, followed by 10 μl of undiluted test compound from the GenPlus library (final concentration of test compound=1 mM). Plates were incubated at 4° C. for about 3-4 hours. Fluorescence was measured in a SpectraMax Gemini plate reader. Cyan fluorescence was measured at excitation 395 nm, emission 475 nm, and cutoff of 455 nm. Yellow fluorescence was measured at excitation 395 nm, emission 529, and cutoff of 515 nm. To determine the degree of FRET occurring in the sample, the cyan fluorescence was divided by yellow fluorescence, and compared to samples with only vehicle (DMSO) present.

[0125] 142 test compounds were identified as being hits with the primary screen (Table 2). These positive hits were from various classes of compounds including flavonoids, dopamine agonist, selenium-containing compounds, etc.

TABLE 2

Test compounds identified as hits.

Compound Name (identified as a hit in the primary screen)	Chemical Structure	Secondary Screen
Cianidanol	flavonoid	No effect (related compound hespiridin had no effect)
Rutoside	flavonoid	Not tested
Quercitin	flavonoid	Not tested
Citropten	flavonoid	Not tested
6,4'-dihydroxyflavone	flavonoid	Not tested
6,7-dihydroxyflavone	flavonoid	Not tested
7,2'-dihydroxyflavone	flavonoid	Not tested
7,3'-dihydroxyflavone	flavonoid	Not tested
7,4'-dihycroxyflavone	flavonoid	Not tested
Naringin	flavonoid	No effect
Metergoline	ergot alkaloids	Not tested
Dihydroergotamine mesylate	ergot alkaloids	Not tested
Ergonovine maleate	ergot alkaloids	Not tested
Methylergonovine	ergot alkaloids	No effect
Aurothioglucose	gold salt	Confirmed
Thimerosal	organic mercury compound	Confirmed
Merbromin	organomercurial	Not tested
Phenylmercuric acetate	organomercurial	Confirmed
Ebselen	novel selenium-containing compound	Confirmed
Cisplatin	platinum-containing compound	Confirmed
Hydrastinine hydrochloride	isoquinoline alkaloid	Not tested
Emetine hydrochloride	isoquinoline alkaloid	Not tested
Berberine	isoquinoline alkaloid	Not tested
Hydrastine	isoquinoline alkaloid	No effect
Amodiaquine	aminoquinolone	No effect
Primaquine phosphate	aminoquinolone	No effect
Amoxicillin	cillin	Not tested
Ampicillin	cillin	Not tested
Hetacillin	cillin	Not tested
Metampicillin	cillin	Not tested
Bacampicillin	cillin	Not tested
Methacycline	tetracycline	Not tested
Meclocycline	tetracycline	Not tested

TABLE 2-continued

Test compounds identified as hits.

Compound Name (identified as a hit in the primary screen)	Chemical Structure	Secondary Screen
Doxycycline	tetracycline	No effect
Chlortetracycline	tetracycline	Not tested
Demeclocycline hydrochloride	tetracycline	Not tested
Minocycline hydrochloride	tetracycline	Not tested
Oxytetracycline	tetracycline	No effect
Tetracycline	tetracycline	Not tested
Anthralin	anthraquinone	Not tested
Danthron	anthraquinone	Not tested
Diacerin	anthraquinone	Not tested
Aloin	anthraquinone	Not tested
Apomorphine	non-ergoline dopamine agonist	Confirmed
R(−)-allylnorapomorphine hydrobromide	non-ergoline dopamine agonist	Not tested
Cephradine sodium	cephalosporin	Not tested (related cefadroxil had no effect)
cefoxitin	cephalosporin	Not tested
Chlorotrianisene	nonsteroidal estrogen	No effect (related compound hexestrol was confirmed)
Dantrolene	nitrofuran derivative	Not tested
Furazolidone	nitrofuran derivative	No effect
Nitrofurantoin	nitrofuran derivative	Not tested
Nitrofurazone	nitrofuran derivative	Not tested
Imipramine	Phenothiazines	Not tested
Propantheline bromide	Phenothiazines	No effect
Propiomazine	Phenothiazines	Not tested
Trifluoperazine	Phenothiazines	Not tested
Flufenazine	Phenothiazines	Not tested
Triflupromazine	Phenothiazines	No effect
Trazodone hydrochloride	Phenothiazines	Not tested
Norepinephrine	adrenergic	Not tested
Isoproterenol hydrochloride	adrenergic	No effect
Levonordefrin	adrenergic	Not tested
Oxidopamine	adrenergic	Not tested
Methotrexate	folic acid	Not tested
Folic acid		No effect
Reserpine	indolealkylamine alkaloid	Not tested
Rescinnamine	indolealkylamine alkaloid	Not tested
Estradiol propionate	estrogen	Not tested (related b-estradiol had no effect)
Estradiol acetate	estrogen	Not tested
Oxolinic acid	quinone	Not tested
Ofloxacin	quinone	No effect
Piroxicam	NSAID - oxicam	Not tested
Tenoxicam	NSAID - oxicam	Not tested
Citrinin	mycotoxin	Not tested
Gentisic acid	aromatic acid	No effect
Veratrine sulfate	cevane	No effect
Amiloride	diuretic (triamterene)	No effect
amphotericin B	polyene	Not tested
Amprolium	thiamine analog	No effect
Bacitracin	metalloantibiotic	Not tested
Benserazide	dopamine agent	Not tested
beta-Carotene	carotenoid	Not tested
chlorhexidine	bisbiguanide antiseptic	No effect
Dipyridamole	pyrimidopyrimidine derivative	Not tested
Epinephrine bitartrate	epinephrines	No effect
Ergocalciferol	vitamin D	Not tested
Gentian violet	triphenylmethane dye	Not tested
Hyydroxyzine pamoate	1st gen histaminergic receptor antagonist	No effect
Norfloxacin	2nd gen quinolones	Not tested (related compounds enoxacin and lomefloxacin had no effect)
Phenazopyridine	other	No effect
Pyrantel pamoate	tetrahydropyrimidines	Confirmed

TABLE 2-continued

Test compounds identified as hits.

Compound Name (identified as a hit in the primary screen)	Chemical Structure	Secondary Screen
Pyrvinium pamoate	other	Not tested
Quinacrine	acridine derivative	Not tested
Roxarsone	arsenic compound	Not tested
Sulfasalazine	other	Not tested
Sulindac	NSAID - indomethacin	No effect
Triamterene	other	Not tested
Tyrothricin	mixture of tyrocidins/gramicidins	Not tested
Acriflavinium hydrochloride	other	No effect
Bergaptene	furocoumarin	Not tested
Rosolic acid	other	No effect
Calcein	fluorescein-iminodiacetic complex	Not tested
Glafenine	anthranilic acid derivative	No effect
Ethoxyquin	quinoline fungicide	Not tested
Fenbendazole	benzimidazole	Not tested
Pimozide	dopamine antagonist	Not tested (related compound droperidol had no effect)
Acecainide hydrochloride	other	Not tested
Erythromycin propionate lauryl sulfate	macrolide antibiotic	No effect
Benzamil hydrochloride	amiloride derivative	Not tested
9-amino-1,2,3,4-tetrahydroacridine hydrochloride	acridine derivative	Not tested
Piromidic acid	Pyridopyrimidines; pyrrolidines; quinolones	Not tested
Alrestatin	quinazoline acetic acid derivatives	Not tested
N-carboxyheptylimidazole hydrochloride	other	Not tested
Anthracene-9-carboxylic acid	anthracene	Not tested
Gossypol-acetic acid complex	other	Confirmed
alpha-cyano-4-hydroxycinnamic acid	other	Not tested
2-phenpropylamino-5-nitrobenzoic acid	other	Not tested
Ellagic acid	tannin; polyphenolic	Confirmed
Aclarubicin	anthracycline	Not tested
Alexidine	bisbiguanide antiseptic	Not tested
Tretinon	retinoic acid	No effect
Cetrimonium bromide	polycationic antiseptic	Not tested
Pararosaniline pamoate	other	Not tested
Nimesulide	sulfoanilide	Not tested
Lupitidine hydrochloride	other	Not tested
Methazolamide	carbonic anhydrase inhibitor	No effect
3,5-dinitrocatechol	catechol derivative	Not tested
Thiram	pesticide	Not tested
Tetroquinone	polyhydroxylated aromatic compound	Not tested
Monensin, sodium	ionophore antibiotic	No effect
Dequalinium chloride	lipophilic cationic compound	No effect
Antimycin A	other	No effect
Mycophenolic acid	other	No effect
3-hydroxymethyl-b-carboline	beta carboline alkaloid	Not tested
Etiocholanolone	androgen	No effect
Lapachol	naphtoquinone	Not tested
Benzalkonium chloride	quatenary ammonium compound	No effect
Trioxsalen	furocoumarin	Not tested (related compound 8-methoxypsoralen)

TABLE 2-continued

Test compounds identified as hits.

Compound Name (identified as a hit in the primary screen)	Chemical Structure	Secondary Screen
Prazosin	cationic drug	No effect
4-naphthalimidobutyric acid	other	Not tested
Metolazone	chemically related to thiazides	Not tested (related compound bendroflumethiazide had no effect)
NPPB	other	Not tested

[0126] Multiple test compounds that were classified as hits in the primary screen as well as additional related compounds were evaluated in a secondary screen designed as follows. Briefly, a 96-well microtiter plate coated with streptavidin (Nunc #436014) was incubated for 2-4 hours at room temperature with 100 µl of a 20-30 µg/ml solution of biotin-tagged PAR6 (whole polypeptide) in phosphate-buffered saline containing Tween-20 (PBST). Plates were washed twice with PBST, then once with incubation buffer (50 mM Tris, pH 8.0; 135 mM NaCl; 10% glycerol; 0.002% EDTA). Incubation buffer (50 µl) was added to each well. The compounds to be tested were added (10 µl) followed by PYN (50 µl) diluted in incubation buffer to an approximate concentration of 4000-5000 relative fluorescent units per 50 µl. Plates were incubated overnight at 4° C. Plates were washed twice with incubation buffer, after which 50 µl incubation buffer was added to retain moisture in the wells. The amount of PYN bound on the plates was determined by measuring yellow fluorescence (532/526) in a Typhoon imager. Fluorescence was quantitated by Softmax Pro software.

[0127] The following test compounds were confirmed via the secondary screen as having the potential to inhibit the interaction of PKC_ι polypeptides with PAR6 polypeptides: aurothioglucose, thimerosal, phenylmercuric acetate, ebselen, cisplatin, apomorphine, pyrantel pamoate, gossypol-acetic acid complex, ellagic acid, and hexestrol. A dose-response analysis was performed using the secondary screening assay and increasing amounts (e.g., 0, 0.1, 1, 10, 100, and 100 µM) of gossypol, hexestrol, thimerosal, ebselen, ATG, ATM, ellagic acid, cisplatin, apomorphine, or phenylmercuric acetate. In each case, a dose-dependent response was detected as the dose increased.

[0128] A dose-response analysis was performed using aurothioglucose (ATG) and aurothiomaleate (ATM) in the FRET assay that was used as the primary screen. Both compounds exhibited a dose-dependent effect on relative binding of PKC_ι and PAR6 polypeptides with less binding being observed as the ATG or ATM concentrations increased (FIG. 18).

[0129] Rac 1 Activity Assays: A549 cells were incubated with the indicated concentration of ATG for one hour prior to analysis. Rac1 activity in A549 cells was assessed by affinity isolation of GTP-bound Rac1 using binding domains of PAK as described elsewhere (Sander et al., *J. Cell Biol.*, 143:1385-98 (1998)). Briefly, cells were lysed in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 20 mM $MgCl_2$, 5 mM EGTA, 10% glycerol, 1% Triton X-100, 1% NP-40, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 µg/ml leupeptin, and 10 µg/ml aprotinin) at

4° C. for 5 min. Cellular debris was removed by centrifugation at 20,000×g for 5 min, and supernatants were transferred to new tubes containing 20 µl of GST-p21-binding domain of PAK1 (PAK1-PBD) coupled to agarose beads (Upstate). An aliquot of each supernatant was reserved to determine total Rac1 and actin expression by immunoblot analysis. Following a 30 minute incubation at 4° C., the agarose beads were collected by centrifugation and washed three times in wash buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 20 mM $MgCl_2$, 5 mM EGTA, 10% glycerol, 1% Triton X-100, 1% NP-40, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 µg/ml leupeptin, and 10 µg/ml aprotinin). Bound polypeptides were solubilized by the addition of 30 µl of SDS sample buffer, resolved by SDS-PAGE, and subjected to immunoblot analysis for Rac1.

[0130] Treatment of cells with ATG resulted in decreased Rac1 activity (FIG. 19). In addition, ATG exhibited a dose-dependent effect on Rac1 activity with less Rac1 activity being observed as the ATG concentrations increased.

[0131] Soft Agar Growth Assays: Anchorage-independent growth was assayed by the ability of cells to form colonies in soft agar. The bottom agar consisted of growth medium containing 10% FBS and 0.75% agarose in 60-mm tissue culture dishes. Nine hundred cells were resuspended in growth medium containing 10% FBS and 0.75% agarose and plated on top of the bottom agar. ATG was added at the indicated concentration to both bottom and top agar solutions. The cells were incubated at 37° C. in 5% CO_2. Cell colonies were visualized and quantified under a dissecting microscope (Olympus) after 4-6 weeks in culture.

[0132] Treatments with 10 µM and 100 µM of ATG resulted in decreased soft agar growth (FIG. 20).

[0133] Tumorigenicity in Nude Mice: The growth of A549 human lung carcinoma cells as established subcutaneous tumors was studied in athymic nude mice (Harlan-Sprague-Dawley, Indianapolis, Ind.) in a defined pathogen-free environment. Briefly, A549 cells were grown in F-12K Nutrient Mixture containing 10% FBS. A549 cells were harvested and resuspended in serum-containing medium. 5×10^6 cells in 100 µl of growth medium were injected subcutaneously into the flank of 4-6 week old female nude mice. Once palpable tumors were established (15 days after inoculation) animals were randomly segregated into two groups. One group received intraperitoneal injections of ATG (200 mg/kg body weight) daily; the second group received an equivalent volume of diluent control solution. Tumor size was measured daily. Tumor growth was quantified by measuring the tumors in three dimensions with calipers. Tumor volume (mm^3) was calculated using the formula: 0.5236 (L×W×H), where L

represents the length of the tumor, W represents the width of the tumor, and H represents the height of the tumor. Animals were individually monitored throughout the experiment.

[0134] Animals treated with ATG exhibited less tumor growth than the tumor growth exhibited in animals treated with saline (FIG. 21). These results demonstrate that test compound identified using the methods and materials provided herein can be used to inhibit PKC$_\iota$ activity and reduce, for example, tumor growth.

Example 8

Animal Model of Lung Cancer

[0135] A tetracycline-based bitransgenic, regulatable expression system has been used to create conditional expression of transgenes specifically in the lung epithelium. Transgenic mice expressing reverse tet transactivator (rtTA) from either the surfactant protein C(SP-C) or Clara Cell Specific Protein (CCSP) promoter allow conditional expression of tet-responsive gene constructs in the lung epithelium. When SP-C-rtTA (or CCSP-rtTA) mice are crossed to transgenic mice expressing a (tetO)7-CMV-transgene, expression of the transgene can be targeted to the lung epithelium under the control of doxycycline. This system has been used to establish the role of oncogenic K-Ras mutations in LAC development and maintenance (Fisher et al., *Genes Dev.*, 15:3249-3262 (2001)). Crossing CCSP-rtTA mice to transgenic mice expressing (tetO)7-CMV-K-RasG12D, generated mice in which oncogenic K-Ras can be conditionally expressed in the lung epithelium by addition of doxycycline to the drinking water. CCSP-rtTA/(tetO)7-K-RasG12D bitransgenic mice develop multiple LACs only after administration of doxycycline. Interestingly, when doxycycline is withdrawn, tumors rapidly regress due to massive apoptosis, showing that K-RasG12D is necessary for both tumor establishment and maintenance.

[0136] Bitransgenic mice were developed to allow conditional expression of kdPKC$_\iota$ in the lung epithelium under the control of doxycycline. To construct this model, transgenic SP-C-rtTA "inducer" mice expressing the reverse tetracycline transactivator protein (rtTA) specifically in the lung epithelium under the control of the SP-C promoter were obtained. In addition, transgenic "responder" mice expressing kdPKC$_\iota$ under the control of a tet responsive promoter, tet(07)-CMV were generated. These mice exhibit germline transmission of a tet(07)-CMV-FLAG-kdPKC$_\iota$ transgene designed to support tet-regulated expression of FLAG-kdPKC$_\iota$. Three independent transgenic tet(07)-CMV-FLAG-kdPKC$_\iota$ mouse lines were established. One of these lines was crossed to SP-C-rtTA mice to establish bitransgenic SPC-rtTA/tet-kdPKC$_\iota$ mice. When bitransgenic SPC-rtTA/tet-kdPKC$_\iota$ mice are given doxycycline in their drinking water, they exhibit tet-regulated expression of FLAG-kdPKC$_\iota$ mRNA in the lung epithelium as determined by QRT-PCR. The kdPKC$_\iota$ transgene was not detected in other tissues (liver, thymus, colon or kidney), indicating that conditional expression is specific to the lung.

[0137] Similar mice can be made to express wild-type PKC$_\iota$ polypeptides or caPKC$_\iota$ polypeptides instead of kdP-KC$_\iota$ polypeptides.

Other Embodiments

[0138] It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

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tatggtttca gtgttgactg gtgggctctt ggagtgctca tgtttgagat gatggcagga     1320

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Cys Ser Phe Asp Asn Glu Gln Leu Phe Thr Met Lys Trp Ile Asp Glu
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Glu Gly Asp Pro Cys Thr Val Ser Ser Gln Leu Glu Leu Glu Glu Ala
65                  70                  75                  80

Phe Arg Leu Tyr Glu Leu Asn Lys Asp Ser Glu Leu Leu Ile His Val
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Phe Pro Cys Val Pro Glu Arg Pro Gly Met Pro Cys Pro Gly Glu Asp
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Lys Ser Ile Tyr Arg Arg Gly Glu Arg Arg Trp Arg Lys Leu Tyr Cys
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                450              455              460

Ile Leu Glu Lys Gln Ile Arg Ile Pro Arg Ser Leu Ser Val Lys Ala
465              470              475              480

Ala Ser Val Leu Lys Ser Phe Leu Asn Lys Asp Pro Lys Glu Arg Leu
                485              490              495

Gly Cys His Pro Gln Thr Gly Phe Ala Asp Ile Gln Gly His Pro Phe
                500              505              510

Phe Arg Asn Val Asp Trp Asp Met Met Glu Gln Lys Gln Val Val Pro
                515              520              525

Pro Phe Lys Pro Asn Ile Ser Gly Glu Phe Gly Leu Asp Asn Phe Asp
                530              535              540

Ser Gln Phe Thr Asn Glu Pro Val Gln Leu Thr Pro Asp Asp Asp Asp
545              550              555              560
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Ile Val Arg Lys Ile Asp Gln Ser Glu Phe Glu Gly Phe Glu Tyr Ile
            565   570   575

Asn Pro Leu Leu Met Ser Ala Glu Glu Cys Val
            580   585

<210> SEQ ID NO 3
<211> LENGTH: 1764
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

atgtcccaca cggtcgcagg cggcggcagc ggggaccatt cccaccaggt ccgggtgaaa   60

gcctactacc gcgggggatat catgataaca cattttgaac cttccatctc ctttgagggc   120

cttttgcaatg aggttcgaga catgtgttct tttgacaacg aacagctctt caccatgaaa   180

tggatagatg aggaaggaga cccgtgtaca gtatcatctc agttggagtt agaagaagcc   240

tttagacttt atgagctaaa caaggattct gaactcttga ttcatgtgtt cccttgtgta   300

ccagaacgtc ctgggatgcc ttgtccagga aagataaat ccatctaccg tagaggtgca   360

cgccgctgga gaaagcttta ttgtgccaat ggccacactt ccaagccaa gcgtttcaac   420

aggcgtgctc actgtccat ctgcacagac cgaatatggg gacttggacg ccaaggatat   480

aagtgcatca actgcaaact cttggttcat aagaagtgcc ataaactcgt cacaattgaa   540

tgtgggcggc attctttgcc acaggaacca gtgatgccca tggatcagtc atccatgcat   600

tctgaccatg cacagacagt aattccatat aatccttcaa gtcatgagag tttggatcaa   660

gttggtgaag aaaaagaggc aatgaacacc agggaaagtg gcaaagcttc atccagtcta   720

ggtcttcagg attttgattt gctccgggta ataggaagag gaagttatgc caaagtactg   780

ttggttcgat taaaaaaac agatcgtatt tatgcaatgt gggttgtgaa aaaagagctt   840

gttaatgatg atgaggatat tgattgggta cagacagaga agcatgtgtt tgagcaggca   900

tccaatcatc ctttccttgt tgggctgcat tcttgctttc agacagaaag cagattgttc   960

tttgttatag agtatgtaaa tggaggagac ctaatgtttc atatgcagcg acaaagaaaa   1020

cttcctgaag aacatgccag attttactct gcagaaatca gtctagcatt aaattatctt   1080

catgagcgag ggataattta tagagatttg aaactggaca atgtattact ggactctgaa   1140

ggccacatta aactcactga ctacggcatg tgtaaggaag gattacggcc aggagataca   1200

accagcactt tctgtggtac tcctaattac attgctcctg aaattttaag aggagaagat   1260

tatggtttca gtgttgactg gtgggctctt ggagtgctca tgtttgagat gatggcagga   1320

aggtctccat ttgatattgt tgggagctcc gataaccctg accagaacac agaggattat   1380

ctcttccaag ttattttgga aaacaaatt cgcataccac gttctctgtc tgtaaaagct   1440

gcaagtgttc tgaagagttt tcttaataag gaccctaagg aacgattggg ttgtcatcct   1500

caaacaggat ttgctgatat tcagggacac ccgttcttcc gaaatgttga ttgggatatg   1560

atggagcaaa aacaggtggt acctcccttt aaaccaaata tttctgggga atttggtttg   1620

gacaactttg attctcagtt tactaatgaa cctgtccagc tcactccaga tgacgatgac   1680

attgtgagga agattgatca gtctgaattt gaaggttttg agtatatcaa tcctcttttg   1740

atgtctgcag aagaatgtgt ctga   1764

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<210> SEQ ID NO 4
<211> LENGTH: 587
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4

Met Ser His Thr Val Ala Gly Gly Ser Gly Asp His Ser His Gln
1               5                   10                  15

Val Arg Val Lys Ala Tyr Tyr Arg Gly Asp Ile Met Ile Thr His Phe
            20                  25                  30

Glu Pro Ser Ile Ser Phe Glu Gly Leu Cys Asn Glu Val Arg Asp Met
        35                  40                  45

Cys Ser Phe Asp Asn Glu Gln Leu Phe Thr Met Lys Trp Ile Asp Glu
    50                  55                  60

Glu Gly Asp Pro Cys Thr Val Ser Ser Gln Leu Glu Leu Glu Glu Ala
65                  70                  75                  80

Phe Arg Leu Tyr Glu Leu Asn Lys Asp Ser Glu Leu Leu Ile His Val
                85                  90                  95

Phe Pro Cys Val Pro Glu Arg Pro Gly Met Pro Cys Pro Gly Glu Asp
            100                 105                 110

Lys Ser Ile Tyr Arg Arg Gly Ala Arg Arg Trp Arg Lys Leu Tyr Cys
            115                 120                 125

Ala Asn Gly His Thr Phe Gln Ala Lys Arg Phe Asn Arg Arg Ala His
        130                 135                 140

Cys Ala Ile Cys Thr Asp Arg Ile Trp Gly Leu Gly Arg Gln Gly Tyr
145                 150                 155                 160

Lys Cys Ile Asn Cys Lys Leu Leu Val His Lys Lys Cys His Lys Leu
                165                 170                 175

Val Thr Ile Glu Cys Gly Arg His Ser Leu Pro Gln Glu Pro Val Met
            180                 185                 190

Pro Met Asp Gln Ser Ser Met His Ser Asp His Ala Gln Thr Val Ile
            195                 200                 205

Pro Tyr Asn Pro Ser Ser His Glu Ser Leu Asp Gln Val Gly Glu Glu
        210                 215                 220

Lys Glu Ala Met Asn Thr Arg Glu Ser Gly Lys Ala Ser Ser Ser Leu
225                 230                 235                 240

Gly Leu Gln Asp Phe Asp Leu Leu Arg Val Ile Gly Arg Gly Ser Tyr
                245                 250                 255

Ala Lys Val Leu Leu Val Arg Leu Lys Lys Thr Asp Arg Ile Tyr Ala
            260                 265                 270

Met Trp Val Val Lys Lys Glu Leu Val Asn Asp Asp Glu Asp Ile Asp
        275                 280                 285

Trp Val Gln Thr Glu Lys His Val Phe Glu Gln Ala Ser Asn His Pro
        290                 295                 300

Phe Leu Val Gly Leu His Ser Cys Phe Gln Thr Glu Ser Arg Leu Phe
305                 310                 315                 320

Phe Val Ile Glu Tyr Val Asn Gly Gly Asp Leu Met Phe His Met Gln
                325                 330                 335

Arg Gln Arg Lys Leu Pro Glu Glu His Ala Arg Phe Tyr Ser Ala Glu
            340                 345                 350

Ile Ser Leu Ala Leu Asn Tyr Leu His Glu Arg Gly Ile Ile Tyr Arg
            355                 360                 365
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Asp Leu Lys Leu Asp Asn Val Leu Leu Asp Ser Glu Gly His Ile Lys
    370                 375                 380

Leu Thr Asp Tyr Gly Met Cys Lys Glu Gly Leu Arg Pro Gly Asp Thr
385                 390                 395                 400

Thr Ser Thr Phe Cys Gly Thr Pro Asn Tyr Ile Ala Pro Glu Ile Leu
                405                 410                 415

Arg Gly Glu Asp Tyr Gly Phe Ser Val Asp Trp Trp Ala Leu Gly Val
                420                 425                 430

Leu Met Phe Glu Met Met Ala Gly Arg Ser Pro Phe Asp Ile Val Gly
            435                 440                 445

Ser Ser Asp Asn Pro Asp Gln Asn Thr Glu Asp Tyr Leu Phe Gln Val
        450                 455                 460

Ile Leu Glu Lys Gln Ile Arg Ile Pro Arg Ser Leu Ser Val Lys Ala
465                 470                 475                 480

Ala Ser Val Leu Lys Ser Phe Leu Asn Lys Asp Pro Lys Glu Arg Leu
                485                 490                 495

Gly Cys His Pro Gln Thr Gly Phe Ala Asp Ile Gln Gly His Pro Phe
            500                 505                 510

Phe Arg Asn Val Asp Trp Asp Met Met Glu Gln Lys Gln Val Val Pro
            515                 520                 525

Pro Phe Lys Pro Asn Ile Ser Gly Glu Phe Gly Leu Asp Asn Phe Asp
        530                 535                 540

Ser Gln Phe Thr Asn Glu Pro Val Gln Leu Thr Pro Asp Asp Asp Asp
545                 550                 555                 560

Ile Val Arg Lys Ile Asp Gln Ser Glu Phe Glu Gly Phe Glu Tyr Ile
                565                 570                 575

Asn Pro Leu Leu Met Ser Ala Glu Glu Cys Val
            580                 585
```

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<210> SEQ ID NO 5
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 5

gcttatgttt gagatgatgg cgg                                            23


<210> SEQ ID NO 6
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 6

gtgacaaccc aatcgttccg                                                20


<210> SEQ ID NO 7
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 7

gtgggccgct ctaggcacca a                                              21


<210> SEQ ID NO 8
<211> LENGTH: 24
<212> TYPE: DNA
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<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 8

ctctttgatg tcacgcacga tttc   24

<210> SEQ ID NO 9
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 9

ggctgcattc ttgctttcag a   21

<210> SEQ ID NO 10
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 10

ccaaaaatat gaagcccagt aatca   25

<210> SEQ ID NO 11
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 11

caatcttacc tgctttct   18

<210> SEQ ID NO 12
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 12

cgttcttccg aaatgttgat tg   22

<210> SEQ ID NO 13
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 13

tccccagaaa tatttggttt aaagg   25

<210> SEQ ID NO 14
<211> LENGTH: 16
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 14

ttgctccatc atatcc   16

27

What is claimed is:

1. A method for inhibiting a protein kinase C iota polypeptide response in a mammal, said method comprising administering an inhibitor to said mammal under conditions wherein said response is inhibited, wherein said inhibitor reduces the interaction between a protein kinase C iota polypeptide and a polypeptide selected from the group consisting of Par-6, Src, Par-4, p62/ZIP, and Par-3 polypeptides.

2. The method of claim 1, wherein said response is cell transformation, development of cancer, or colon carcinogenesis.

3. The method of claim 1, wherein said inhibitor is a polypeptide fragment.

4. The method of claim 3, wherein said polypeptide fragment comprises an amino acid sequence present in said protein kinase C iota polypeptide.

5. The method of claim 1, wherein said inhibitor is aurothioglucose, aurothiomaleate, thimerosal, phenylmercuric acetate, ebselen, cisplatin, apomorphine, pyrantel pamoate, gossypol-acetic acid complex, ellagic acid, or hexestrol.

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