DECIPHERING HOW RETINOL INHIBITS METASTASIS THROUGH PHOSPHATIDYLINOSITOL 3-KINASE

THESIS

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I INTRODUCTION AND LITERATURE REVIEW

COLORECTAL CANCER

Colorectal cancer (CRC) is the third most common cancer and third leading cause of cancer deaths in the United States. In 2011, the American Cancer Society predicted the US will see 141,000 new cases of colorectal cancer, as well as nearly 50,000 related deaths (American Cancer Society 2011; Edwards et al. 2010). In addition, incidence rates of CRC have been increasing steadily among adults younger than the recommended screening age of 50 years, by about two percent per year since 1994 (American Cancer Society 2011). Globally, the rate of CRC incidence is rising in developing nations due to increased exposure to risk factors (Center et al. 2009). Though controversial, the exploration of natural therapies as treatments for cancer has attracted attention due to their decreased cost and potentially higher safety when compared to pharmaceuticals. Of all drugs approved by the FDA to combat cancer in the past 30 years, 70% are reported to stem from natural compounds or can be categorized as traditional medicine (Collins and Workman 2006; Cragg et al. 2009).

Metastasis

Death due to CRC generally results from cancer metastasis to sites outside the colon, rather than from the primary tumor itself. The most frequent site of CRC metastasis is the liver (Galandiuk et al. 1992; Scheele et al. 1995; Tominaga et al. 1996). Survival rates for all stages of CRC have increased in recent years to as high as 90% at five years with only localized disease. The survival rate drops to only 12% for people presenting with distant metastases (Edwards et al. 2010). Metastasis is facilitated by cell signaling cascades that have been rendered abnormal due to genetic mutations.

Mutations that occur in specific genes create abnormal states of activation or inhibition in key protein signalers, which in turn spurs on the survival, propagation, and motility of tumor cells. In various types of cancer, one protein often affected is phosphotydlinositol-3 kinase (PI3K). The activation of PI3K in turn affects downstream signalers including 3-phosphoinositide-dependent protein kinase (PDK-1), protein kinase B (Akt), activator protein 1 (AP-1), and nuclear factor κ-light-chain-enhancer of activated B cells (NFκB). The result of this abnormal cascade is increased expression of a key metastatic enzyme, matrixmetalloproteinase-9 (MMP-9), and ultimately increased metastasis.

PI3K SIGNALING

PI3K catalyzes a signaling cascade which ultimately results in the activation of oncogenic transcription factors AP-1 and NFκB, causing increased expression of MMP-9. In the interim, several other proteins are activated including PDK-1, and subsequently Akt (Fig. 1). In addition, pathways otherwise considered separately (MEK and mTOR signaling cascades, for example) interact with the PI3K cascade with the activation of overlapping proteins such as extracellular signal-regulated kinases 1 and 2 (ERK) and mammalian target of rapamycin (mTOR). The activation of all these proteins may serve to perpetuate the signaling cascade initiated by PI3K, with a purported secondary role of mTOR as a negative regulator elsewhere within the pathway (See McCubrey et al. 2011, for review). The intermingling of pathways involving PI3K/Akt, MEK/ERK, and mTOR signaling is discussed further in Chapter 4. To our knowledge, other than the current work, the effect of retinol on PDK-1 activity as well as AP-1 and NFκB mediated gene-transcription has not been examined, though studies involving other retinoids are discussed in Chapter 3.

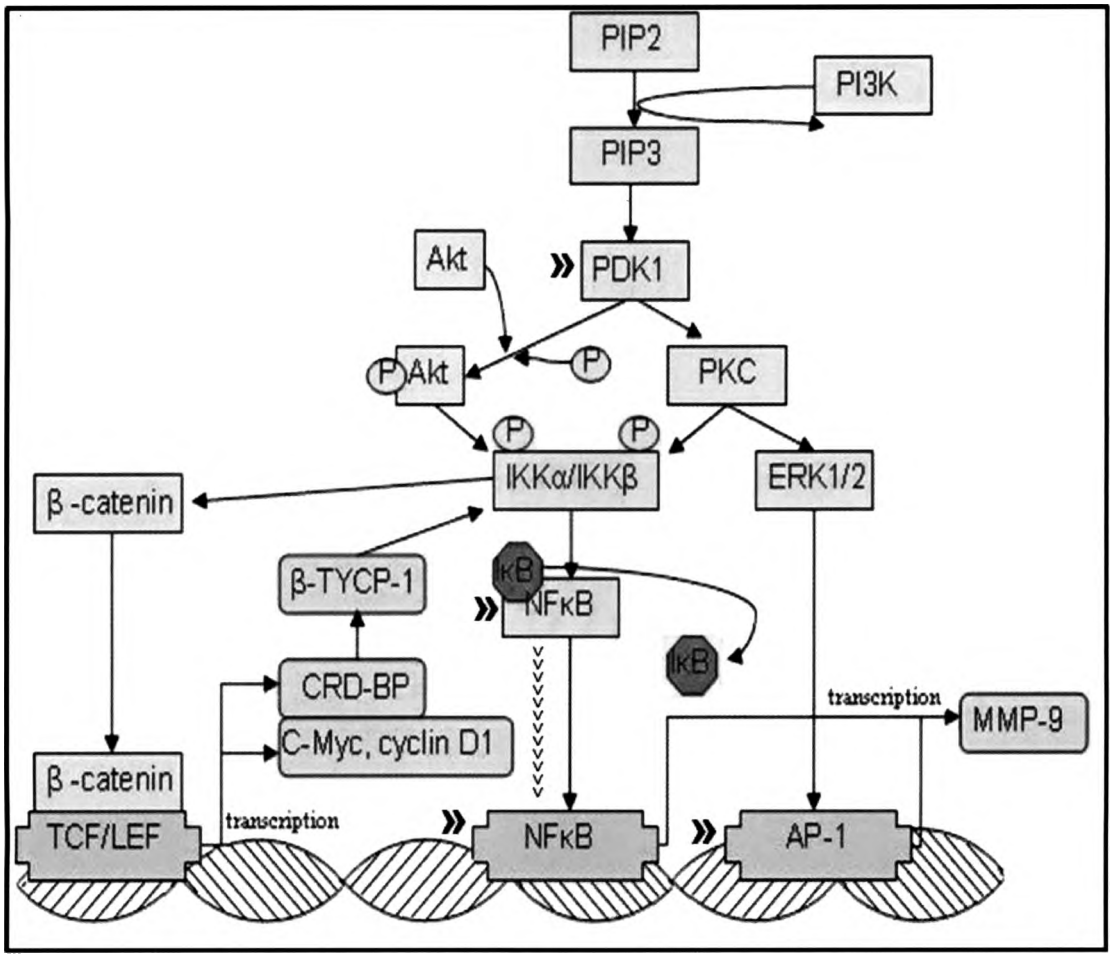

Fig. 1 The PI3K Pathway. A schematic representation of basic protein interactions progressing throughout the PI3K signaling cascade, simplified to emphasize proteins studied in this work and relation to MMP-9. PDK1, NFκB, and AP-1 were examined as potential targets for retinol mediation. Results are discussed in Chapter 3.

PI3K Activation and Cancer

PI3K has a key role in the regulation of many cellular processes including proliferation, cell survival, carbohydrate metabolism, and motility (Stein et al. 2001). The gene encoding PI3K (PIK3CA) is somatically mutated in over 25% of colorectal tumors resulting in upregulated activity, and amplification of genomic regions containing genes encoding pathway proteins have also been reported (Samuels et al. 2005). Activation of PI3K is associated with increased cell invasion and tumor metastasis (Keely et al. 1997; Stephens et al. 2005). Through a series of knockout experiments, Ericson et al. (2010) demonstrated that PI3K signaling is "wired differently in human cancer cells than in other cell types or organisms". Class 1A PI3Ks, the class expressed in human colon cancers, are heterodimers composed of p110α catalytic and p85 regulatory subunits. PI3K is recruited to the cell surface and activated by growth factor receptor tyrosine kinases (such as the insulin receptor following activation by insulin) (Raynaud et al. 2007). Active PI3K catalyzes the phosphorylation of the 3-OH position of the inositol head group of phosphatidylinositol 4,5 bisphosphate (PIP2), converting its substrate to phosphatidylinositol 3,4,5-triphosphate (PIP3) (Samuels et al. 2005; Fig. 2).

Previously, we reported that retinol treatment reduces the activity of PI3K (Park et al. 2008; Fig. 3). This effect was dose-responsive and independent of RAR- and ATRA -mediated gene transcription. In addition, we recently completed a series of experiments showing that expression of constitutively active (ca) PI3K in human CRC cells blocks the ability of retinol to decrease cell invasion (Fig. 4; Griffin and Lane, manuscript in preparation). This same phenomenon occurred *in vivo*, when caPI3K-

expressing CRC cells were intrasplenically injected into nude mice (Fig. 5; Griffin and Lane, manuscript in preparation). Together, these data indicate that the effect of retinol on colon cancer invasion is mediated by PI3K.

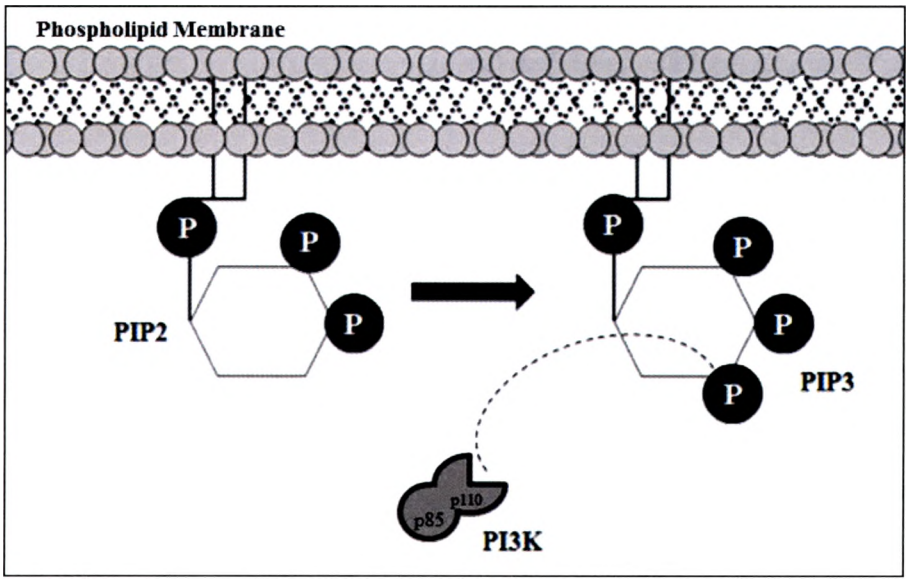

Fig. 2 Generation of PIP3 by PI3K. The catalytic unit of the PI3K heterodimer, p110, phosphorylates the third carbon of the phosphatidylinositol 4,5 bisphosphate (PIP2) ring, generating a second messenger molecule and perpetuating the cascade downstream.

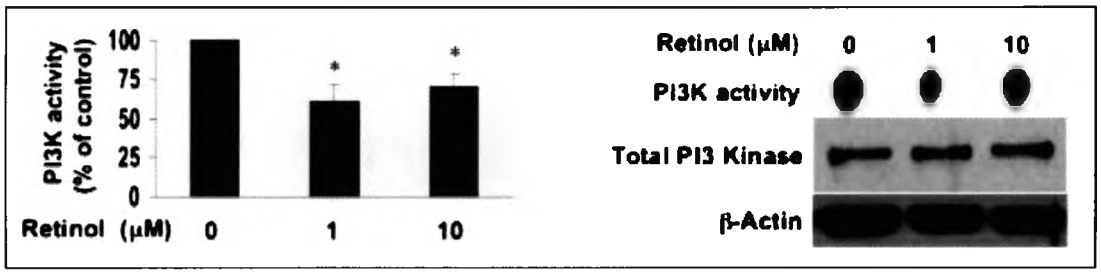

Fig. 3 (From Park et al. 2008) Retinol Decreases PI3K Activity. Total protein was isolated from HCT-116 cells treated with 0 (ethanol vehicle control), 1, or 10 μM retinol for 30 min after 24 h of serum starvation. PI3K was immunoprecipitated using IRS-1 antibody. Immunoprecipitated PI3K was incubated with PI for 10 min followed by incubation with [gamma-32P] ATP for 20 min. Following incubation, the lipids were extracted and separated by TLC. PI3K activity was normalized to total PI3K protein. Total PI3K protein was detected using p85 antibody and normalized to β-actin. Data are reported as mean ± SEM for n=3. *Significantly different from control; $P < 0.05$

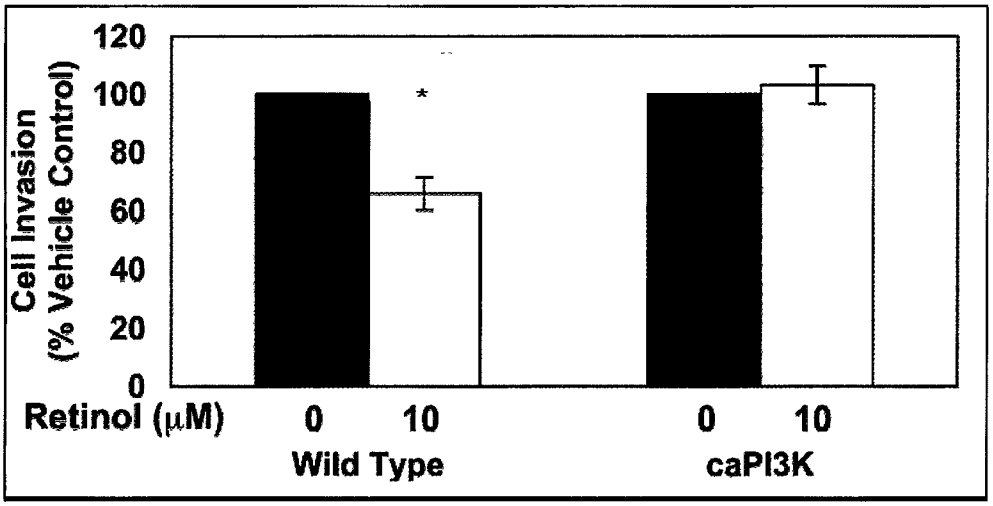

Fig. 4 (From Griffin and Lane, Manuscript in Preparation) caPI3K Blocks the Ability of Retinol to Decrease Cell Invasion. HCT-116 cells expressing two alleles of caPI3K were obtained from Bert Vogelstein. Cell invasion was expressed as % vehicle control, mean ± SEM, n=3. *Significantly different from control; $P < 0.05$

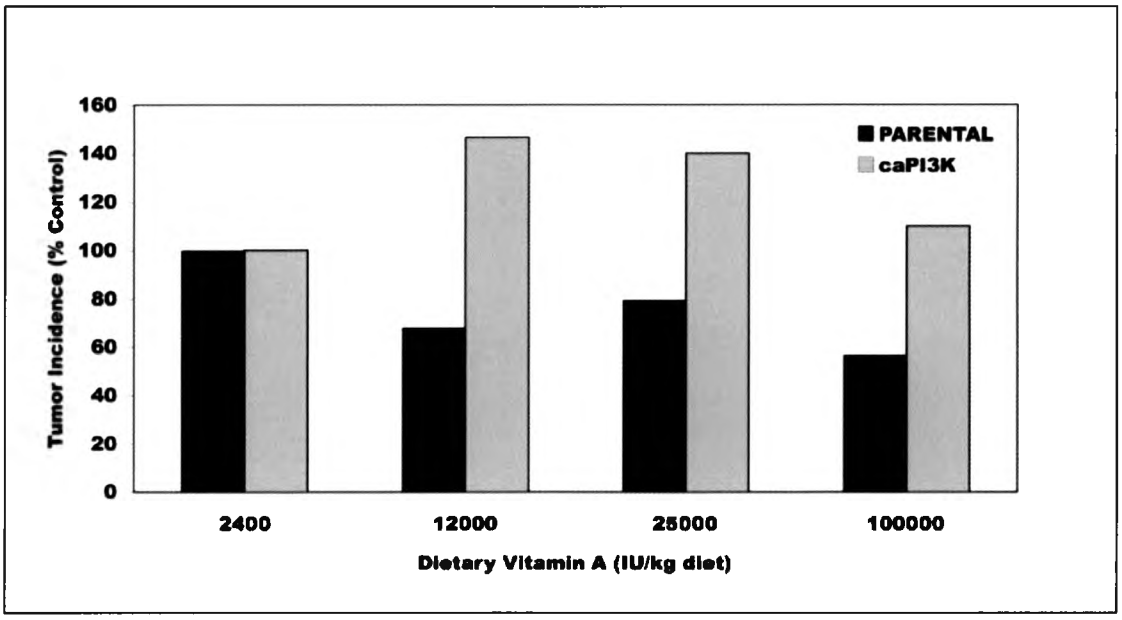

Fig. 5 (From Griffin and Lane, Manuscript in Preparation) Expression of caPI3K Blocks the Ability of Retinol to Decrease CRC Metastasis *in vivo*. Livers were assessed for metastatic incidence. The presence of human colon cells was verified by immunohistochemistry using hematoxylin/eosin and cytokeratin 20 staining. Data shown are percent of mice injected with parental or caPI3K HCT-116 cells that were positive for metastasis. Data are expressed relative to control diet (2400 IU vitamin A/kg diet).

AP-1

AP-1 is activated by a number of cellular signals to produce a number of outcomes. When the transcription factor is activated via PI3K and similar signaling pathways, AP-1 drives the transcription of inflammatory and metastatic proteins. Among these is the enzyme MMP-9, which has been found to upregulated by activated AP-1 in many types of cancer (Inoue et al. 2007; Weng et al. 2008). Retinoids have been shown to affect the ability of AP-1 to drive transcription by interacting with binding proteins on particular DNA response elements. This is the reported mechanism behind reduced proliferation seen following retinoid treatment (Chen et al. 1995). Typically, studies exploring the relationship between retinoids and AP-1 tend to focus on the activation of nuclear retinoic acid receptors (RARs) in response to a retinoid ligand.

NFκB

When stimulated by the PI3K cascade, NFκB acts upon genes to promote cell survival and inhibit apoptosis by inducing or suppressing the transcription of different proteins (Lee et al. 2007). However, NFκB may demonstrate the ability to promote apoptosis in response to other signaling systems under certain conditions (Zanotto-Filho et al. 2009). Notably, NFκB also regulates the expression of metastatic proteins including MMP-9. Studies have shown that blocking NFκB activity sufficiently inhibits MMP-9 expression, cell invasion, and metastasis in colorectal cancer as well as several other cancer types (Huang et al. 2001; Lu et al. 2010; Park et al. 2009; Schwab 2000; Seo et al. 2009; Yang et al. 2010). NFκB levels are higher in metastatic tumor cells than in non-metastatic cells, and increased activity of this transcription factor has been shown to promote the metastasis in some of these tissue types (Yan et al. 2010). Like AP-1, it is

theorized that NFκB is inhibited from transcribing MMP-9 and other MMP proteins following retinoid treatment due to retinoid-binding and activation of RARs (Na et al. 1999).

MMP-9 and Metastasis

MMP-9 is an extracellular enzyme that has been widely studied due to its role in cell migration and metastatic progression of colorectal cancer (Bergers et al. 2000; Bernhard et al. 1994; Legrand et al. 1999; Huang et al. 2002; McCawley et al. 2001). MMP-9 is a gelatinase capable of degrading the type IV collagen associated with basement membranes and found throughout the colonic mucosa. Degradation of this structural tissue is closely related to cell invasion and metastasis and is how MMP-9 facilitates the movement and re-establishment of tumor cells outside the colon (Roskell 1998; Zeng et al. 1999). In addition, several studies have reported MMP-9 as a downstream target of PI3K/Akt activation and a key protein responsible for invasion in various carcinomas (Arcaro et al. 2007; Cheng et al. 2006; Chen et al. 2009). It is purported that the over-expression of MMP-9 observed in colorectal carcinomas may be part of the mechanism by which carcinoma cells metastasize (Heslin et al. 2001). Increased levels of MMP-9 mRNA in colorectal cancer compared with normal mucosa have been associated with significantly shorter disease-free and overall survival (Zeng et al. 1996). In addition, a higher incidence of MMP-9 expression occurs in colorectal tumors when liver metastases are present (Koumura et al. 1997). Reports have shown patients with colon carcinoma have significantly increased levels of MMP-9 protein and up-regulation of MMP-9 transcription in tumor areas compared with regions of normal tissue (Roeb et al. 2001). Induced expression of MMP-9 by the hepatitis B

virus X protein (HBx) in hepatocellular carcinoma cells was reduced by inhibition of the PI3K pathway, indicating that PI3K signaling is involved in transcriptional regulation of MMP-9 (Chung et al. 2004). This notion is further supported by the recent report that activation of the PI3K/Akt signaling pathway correlated with frequent intrahepatic metastasis and vascular invasion through up-regulation of MMP-9 expression in human hepatocellular carcinoma (Chen et al. 2009).

Our laboratory has demonstrated that retinol treatment reduced invasion of HCT-116 cells by increasing the tissue inhibitors of matrix metalloproteinase 1 (TIMP-1) protein, as well as decreasing MMP-9 mRNA, protein, and activity levels (Park et al. 2007; Fig. 6). Additionally, we found that cell invasion was significantly decreased in HCT-116 cells when MMP-9 activity was inhibited using neutralizing antibodies (Park et al. 2007). These data show that MMP-9 plays a role in HCT-116 cell invasion and that retinol decreases invasion in this model partially due to inhibition of MMP-9 activity. We have also shown that reduced MMP-9 levels and cell motility is partially attributed to retinol's inhibition of Akt, generally upregulated in response to PI3K activation in CRC cells (Griffin and Lane, manuscript in preparation; Park et al. 2008). Retinoids reduce invasion and metastasis by decreasing MMP protein levels. For example, ATRA reduced breast cancer cell invasion by decreasing MMP-9 activity (Liu et al. 2003). Also, ATRA has been shown to decrease MMP-9 and increase TIMP-1 gene expression in murine lung alveolar carcinoma (Andela and Rosier 2004). Treatment with the retinoids 6-OH-11-O-hydroxyphenantrene (HF) or ATRA decreased the invasive ability of glioblastoma U87MG cells as well as markedly decreased MMP-9 expression (Papi et al.

2007). However, tumors frequently become ATRA resistant and the effects of ATRA are diminished as cancer progresses.

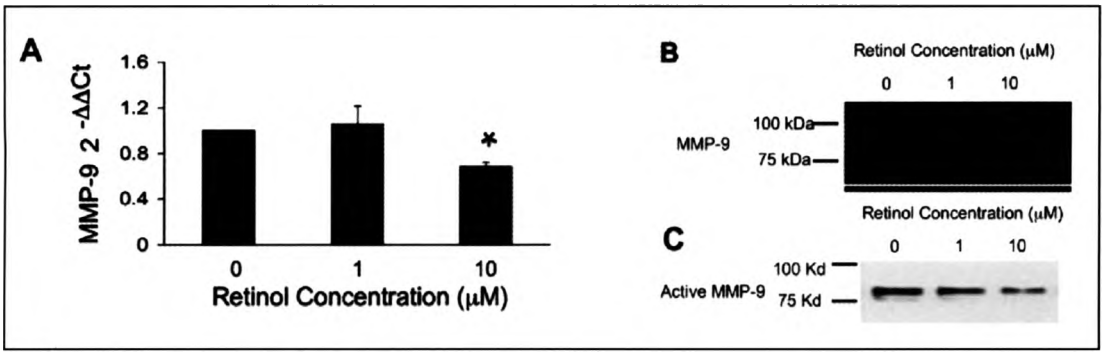

Fig. 6 (From Park et al. 2007) Retinol Decreases MMP-9 mRNA, Activity, and Protein Levels *in vitro*. HCT-116 cells were treated with 0 (ethanol vehicle control), 1, or 10 μM retinol for 24 h. (A) MMP-9 mRNA levels were detected by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Quantitative real-time RT-PCRs were performed in duplicate. Relative amounts of MMP cDNA were calculated by the comparative CT method. CT values were normalized to CT values of GAPDH. Data shown are mean ± SEM, n=3. *Significantly different from control; $P<0.05$. (B) Representative gelatin zymogram displaying the active form of MMP-9 is shown (for n=3). (C) Western blot displaying active MMP-9 protein levels in serum-free conditioned media harvested from cells treated for 24 h with retinol (for n=3).

RETINOIDS

Retinoid Metabolism

The retinoids are a group of vitamin A-related compounds that consist of vitamin A (retinol), its natural metabolites (ATRA), and several synthetic compounds. Some retinoids have been shown to act as cancer chemopreventive agents (Brtko 2007; Marquez et al. 2010; Okuno et al. 2004; Shen et al. 2004). Vitamin A is obtained in the diet from plant sources in the form of pre-vitamin A carotenoids, found in plants, or as preformed vitamin A (retinol or retinyl-esters) in animal-derived food sources. To enable absorption, retinyl-esters are cleaved to form retinol within the intestinal lumen. Retinol is then re-esterified following absorption and incorporated into chylomicrons, which enter the lymphatic system. While in circulation, chylomicrons distribute their contents to peripheral cells before being taken up by the liver, the primary vitamin A storage organ, as chylomicron remnants. Colonocytes receive retinol from the blood either bound to retinol-binding protein (RBP) or as free retinol (Harrison and Hussain 2001) in addition to dietary retinol from the intestinal lumen. Serum retinol generally remains within the range of 1-2 μM in non-deficient animals regardless of vitamin A intake (Ross 1999), while elevated concentrations of retinol within the liver and intestinal lumen have been achieved by dietary vitamin A supplementation (Garcia et al. 2005).

Retinoids and Metastasis

Previous studies evaluating the chemopreventive and chemotherapeutic potential of retinoids have largely focused on various isoforms of retinoic acid (RA); including ATRA, 9-*cis*-RA, and 13-*cis*-RA; or have utilized a synthetic retinoid such as 4-(hydroxyphenyl)retinamide (4-HPR). ATRA, a natural metabolite of retinol, has been

studied most extensively (Mangiarotti et al. 1997; Bartolini et al. 2004; Dutta et al. 2009; Hoffman and Mielicki 2010). Because diminutive amounts of ATRA are found in the diet, human colonocytes are exposed to vitamin A present in the intestinal lumen and in circulation primarily in the form of retinol. Therefore, studies utilizing exogenous ATRA as a model to determine the effects of vitamin A are questionable in both physiological relevance, as ATRA is not consumed nor circulated systemically but rather produced and used locally within cells, and in the assumption that ATRA is solely responsible for all biological effects attributed to retinol.

Within enterocytes and most other cells, retinol is converted to retinyl-esters for storage or metabolized to ATRA for immediate utilization. ATRA functions as a transcriptional regulator, binding to retinoic acid receptors (RARs) located in cell nuclei to inhibit cell growth and differentiation. RARs heterodimerize with retinoid "X" receptors (RXRs) and bind to retinoic acid response elements (RAREs) located in the regulatory regions of retinoid-responsive genes. When ATRA binds to the RAR member of the RAR/RXR heterodimer, gene transcription via RARE is induced (Soprano et al. 2004). Interestingly, ATRA induces the expression of the RAR through this mechanism, sensitizing the cell to the growth-inhibitory effects of ATRA (Altucci et al. 2007; Mellinghoff and Sawyers 2002; Okuno et al. 2004). ATRA-resistance, or the inability of ATRA to inhibit cell growth and induce differentiation, occurs as cancer progresses due to loss of RAR expression following methylation of the RARE in the RAR promoter (Soprano et al. 2004). Research in our laboratory focuses on the ability of retinol and dietary vitamin A to inhibit the growth and metastasis of ATRA-resistant CRC (Dillard et

al. 2007; Park et al. 2005; Park et al. 2007; Park et al. 2008). The work presented here focused on metastasis.

Retinoids have been shown to inhibit metastasis in a variety of model systems. For example, dietary retinyl palmitate decreased malignant melanoma metastasis in mice (Weinzweig et al. 2003), retinol similarly decreased hepatic metastases in a hamster model of pancreatic ductal carcinoma (Heukamp et al. 2005), and in addition, treatment with a combination of cisplatin (chemotherapy drug) and 13-*cis*-RA resulted in a significant reduction in primary tumor size and the number of lung metastatic nodules in murine melanoma B16-F10 cells and melanoma-bearing mice (Liu et al. 2008). Previous research in our laboratory found that both cell migration and invasion were inhibited by retinol in a dose-responsive manner (Park et al. 2005; Fig. 7). Treatment with cyclohexamide, actinomycin D, and a RAR-specific antagonist did not block the inhibitory effect of retinol on cell invasion (Park et al. 2007). These data indicate that the effects of retinol are ATRA- and RAR-independent. Additionally, the activity of PI3K, a crucial enzyme in CRC metastasis (Stein et al. 2001), was diminished in a dose-responsive manner without an effect on amount or heterodimerization of both enzyme subunits: p110 and p85 (Park et al. 2008).

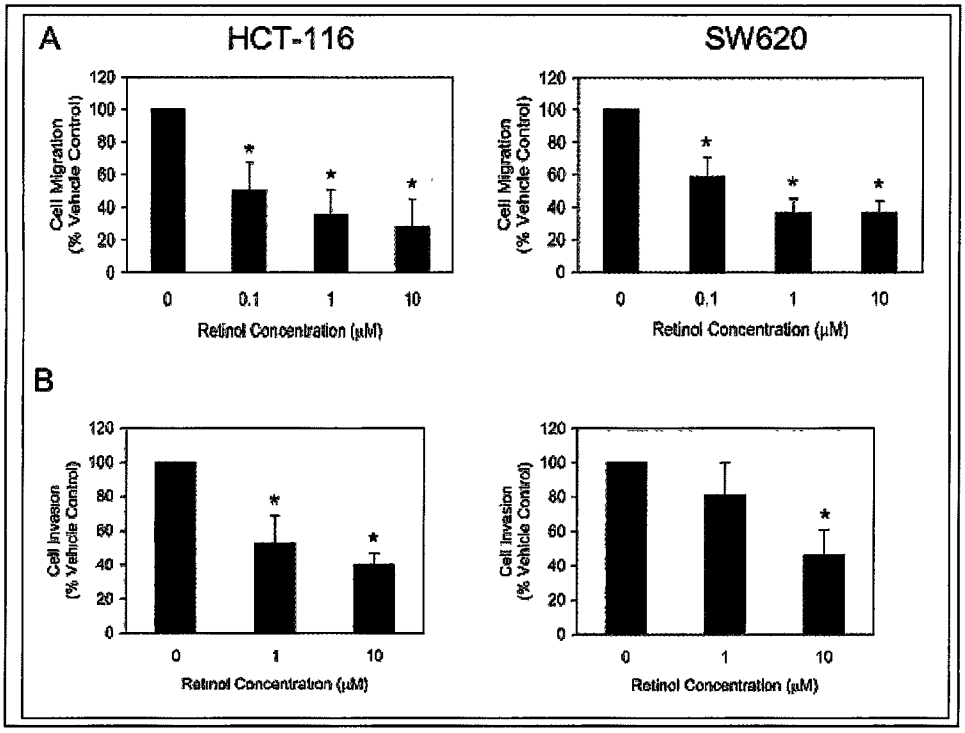

Fig. 7 (From Park et al. 2005) Retinol Decreases Cell Migration and Invasion of ATRA-resistant Colon Cancer Cell Lines. HCT-116 (left column) and SW620 (right column) cells were serum starved for 48 h before seeding at a density of 1 x105 cell per well. (A) Uncoated Boyden chambers were used to assess the effect of retinol on cell migration. The upper portion of the chambers contained 0 (ethanol vehicle control), 0.1, 1, or 10 μM retinol. An 8-μm pore-sized filter separated the cells from a lower chamber containing 10% fetal bovine serum (FBS), which served as a chemoattractant. Cell migration was measured after 8 h by propidium iodide staining. All data are reported as mean +/- SE for three (HCT-116) or five (SW620) experiments. (B) Matrigel-coated Boyden chambers were used to examine the effect of retinol on cell invasion. The upper portion of the chambers contained 0 (ethanol vehicle control), 1, or 10 μM retinol and the lower portion contained 20 ng/mL hepatic growth factor and 10% FBS, which served as chemoattractants. Cell invasion was measured after 24 h by propidium iodide staining as described in Park et al. 2005. All data are reported as mean ± SE for n=3. *Significantly different from control; $P < 0.05$.

RETINOL-PI3K INTERACTION

PI3K is comprised of two protein subunits: p110 and p85. p110 contains an N-terminal region that interacts with regulatory protein subunits, a domain that binds a small G protein Ras (upstream regulator of MEK/ERK signaling), a PIK domain, and a C- terminal catalytic subunit (the domains of p110α are displayed in Chapter 2, Fig. 9). The p85 regulatory unit does not catalyze kinase activity but contains modular domains that can be separated functionally and spatially from the rest of the protein (Fruman et al. 1998). PI3K conducts two main phosphorylation activities. Firstly, autophosphorylation, occurring when the p110 subunit phosphorylates p85 at serine 608, results in inhibition of p110α lipid kinase activity. Secondly, lipid phosphorylation (of phosphatidylinositol (PI) membrane lipids in particular), passes along a signal in an intracellular signaling cascade. Wortmannin irreversibly inhibits PI3K's lipid and protein kinase activities by reacting covalently with lysine-802 in the ATP binding site of the p110α subunit (Ihle and Powis 2010).

Comparison of the electrostatic potential surfaces of retinol, PI, and the PI3K inhibitor wortmannin, revealed retinol (with a ring-shaped tail) had a more similar electrostatic potential surface profile to wortmannin than to PI (Park et al. 2008; Fig. 8). Previous work has shown that wortmannin interacts with the ATP binding site of the p110α catalytic subunit of PI3K to inhibit activity (Wymann et al. 1996), specifically by interacting covalently with lysine-802 (Ihle and Powis 2010). Recently, we used a liquid phase ligand/protein interaction model, in collaboration with Dr. Jon Robertus at UT, Austin, to determine how retinol interacts with the p110α subunit of PI3K. Our modeling

showed that retinol interacts with p110α at the ATP binding site of PI3K, similar to wortmannin. However, unlike the ring-tail formation seen in the previous gas-phase modeling, retinol appeared to interact with a linear tail conformation in the liquid phase model (seen in Chapter 2, Fig. 10). Therefore, it is likely that retinol interacts with PI3K in a manner similar to that of wortmannin. Chapter 2 discusses our use of binding assays aimed to determine if retinol binds PI3K and further validate previous computer modeling studies.

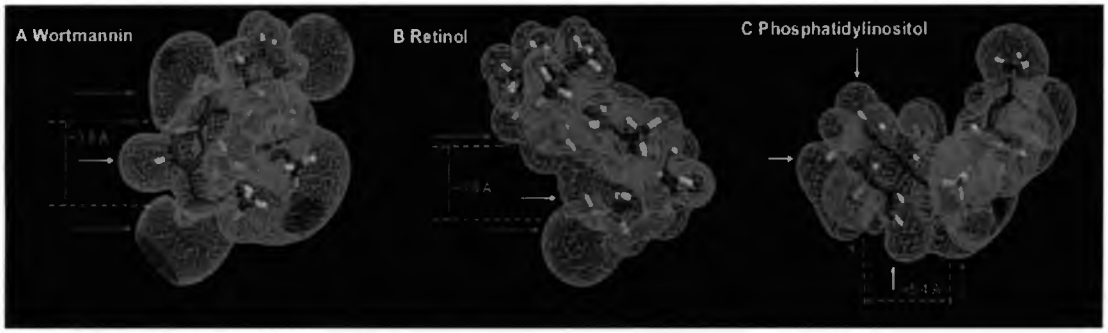

Fig. 8 (From Park et al. 2008) Retinol and Wortmannin Exhibit Similar Electrostatic Potential Surfaces. Electrostatic potential surfaces for (A) wortmannin, (B) retinol, and (C) a PI fragment were calculated as described in Park et al., 2008. In all three panels purple wire frames indicate areas of negative electrostatic potential whereas green indicates positive potential. Surfaces are shown over a tube structure for the conformation with carbon atoms in blue, oxygen atoms in red, hydrogen atoms in white and phosphorus atoms in yellow. Arrows indicate areas associated with either reactive hydrogen atoms (white) or moieties responsible for largely negative electrostatic potential (red and blue).

II RETINOL APPEARS TO BIND PI3K

ABSTRACT

The p110 subunit of PI3K is responsible for PI3K's catalytic activity. Retinol inhibits PI3K activity and computer modeling has shown that retinol and PI3K inhibitor, wortmannin have similar electrostatic characteristics. Also, modeling indicates that retinol appears to insert into the ATP binding site of p110α. Our objective was to determine if retinol binds to the p110α subunit of PI3K. [^{3}H]-Retinol was added to 200 nM and 400 nM concentrations of purified p110α protein with or without 100-fold excess unlabeled retinol. The same procedure was carried out using the labeled p110α substrate, [^{3}H]-phosphatidyl inositol. Binding was measured by scintillation. Our data indicate that retinol does indeed bind to p110α. Future studies in our laboratory will use competitive binding assays to elucidate the exact region where retinol-p110α interaction takes place.

INTRODUCTION

Two proteins make up the functionally diverse subunits of PI3K. p85 serves as a regulatory subunit governing p110 catalytic activity, while p110 is responsible for PI3K substrate interactions (Ihle and Powis 2010). The gene coding for p110α is one of the most frequently mutated genes in human cancers. Alterations to this gene most often occur at the site of p110α/p85 articulation or at points of interaction between the kinase domain and other domains within the catalytic p110 subunit (Huang et al. 2007; Fig. 9). Wortmannin inhibits PI3K by binding covalently with lysine-802 in the catalytic region (also the ATP binding site) of the p110α subunit (Ihle and Powis 2010). We hypothesize that retiol inhibits PI3K similarly because retinol and wortmannin have similar electrostatic profiles. (Park et al. 2008; shown in Chapter 1, Fig. 8) Also, liquid phase modeling indicates that retinol also interacts with p110α at the ATP binding site (Fig. 10). Our objective was to determine if retinol binds to p110α as these models suggest.

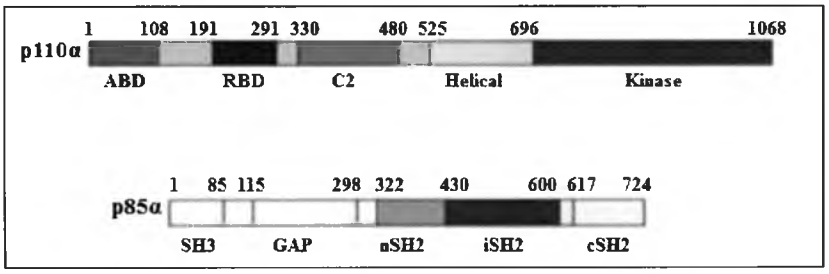

Fig. 9 (Adapted from Huang et al. 2007) Subunits of PI3K. Illustrated are the domains and linking regions as they appear on p110α and p85α. Five domains comprise p110α: the ABD (adaptor binding domain) that binds to p85, the Ras binding domain (RBD), the C2 (proposed to bind to cell membranes), the helical domain (function unknown), and the kinase catalytic domain.

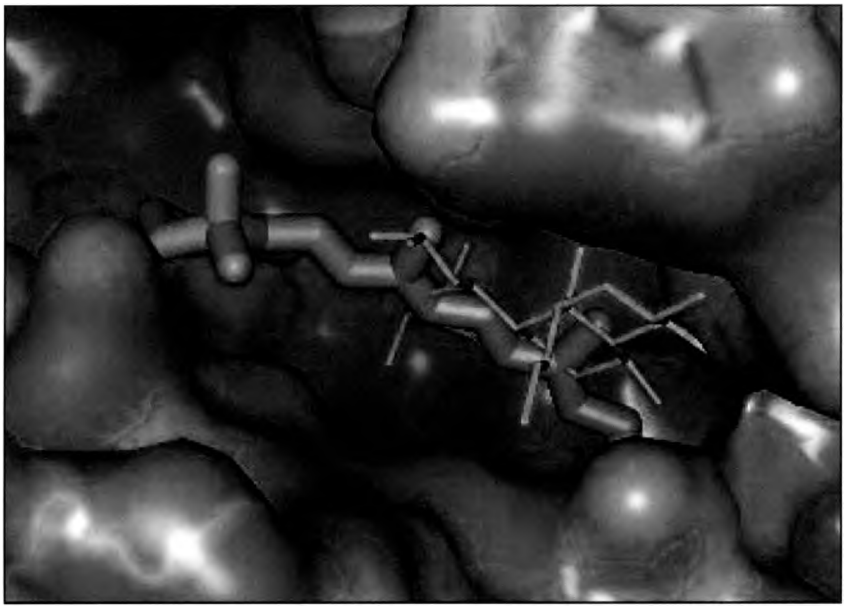

Fig. 10 Computer Modeling of Retinol Ligand-binding to p110α. Liquid phase modeling shows retinol binding to the ATP-binding/catalytic domain of p110α. In contrast to the gas phase modeling shown in Fig. 8, retinol is shown here interacting with a straight-tail formation.

MATERIALS AND METHODS

Western Blot Analysis and Immunoprecipitation

Parental HCT-116 cells were obtained from the ATCC (Manassas, VA) and cultured as in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics at a concentration of 1% at 37°C in a 5% CO_2, humidified atmosphere. Following cell lysis, protein was quantified with the BioRad DC protein assay kit (Hercules, CA).

Magnetic Protein A Sephorose Beads (GE Life Sciences, product # 28-9670-56, Piscataway, NJ) were prepared and crosslinked with p110α monoclonal antibody (Cell Signaling Technology, catalogue #4249, Danvers, MA) using the Protein A/G SpinTrap Buffer Kit (GE Life Sciences, product # 28-9135-67, Piscataway, NJ) according to the "Crosslink" protocol supplied by the manufacturer. Specifically, beads were incubated with antibody and supplied Binding Buffer at 4°C with end-over-end mixing for 15 min. Beads were washed with fresh Binding Buffer and further incubated with the supplied Crosslink Solution A (500 µl triethanolamine) and 50 mM DMP for another 60 min. Following another wash, beads were blocked with Crosslink Solution B (500 µl ethanolamine) for 15 min and the liquid discarded. Beads were then washed with 500 µl of supplied Elution Buffer which was used to discard any unbound antibody. Altogether, 15,000 µg of protein from cell lysate was incubated with 150 µg p110 antibody and 1,250 µl beads in 4°C with end-over-end mixing overnight. Following elution (done twice), isolated protein was divided equally among samples for binding assay.

To verify that p110α immunopreciptiation was successful, 50 μg of protein were electrophoresed through a 10% SDS–PAGE gel before transfer to a nitrocellulose membrane. The membrane was blocked with 5% BSA in 1X TBST (10 mM Tris, pH 8, 150 mM NaCl, and 0.1% Tween-20) for 1 h at room temperature before incubation with monoclonal p110α antibody (Cell Signaling Technology, catalogue #4249, Danvers, MA) at a 1:1000 dilution overnight in 4°C. Immunoreactivity was detected with the Pierce Horseradish Peroxidase Super Signal West Pico Chemiluminescent Substrate kit (Rockford, IL) and chemiluminescence measured using Fotodyne Image Analyzer and transilluminator (Hartland, WI).

Retinol Binding Assay

To measure the ability of [^{3}H]-retinol (Perkin-Elmer, Waltham, MA) to bind to p110α, protein was incubated with 2 mM dithiothreitol, 10 mM Tris buffer (pH 7.4), and 200-400 nM [^{3}H]-retinol either alone or with 100-fold molar excess cold retinol (dissolved in 100% ethanol) for 5 h at 4°C. The excess unlabeled retinol was used to determine nonspecific [^{3}H]-retinol binding, and the volume of ethanol vehicle did not exceed 4% of the total incubation volume. Excess [^{3}H]-retinol was extracted using 100 μl of a suspension of 2.5% charcoal and 0.2% dextran-sulfate. Charcoal was removed by centrifugation and 100 μl of supernatant was counted by liquid scintillation. The procedure was repeated using labeled p110α substrate, [^{3}H]-phosphatidyl inositol (PI), as a positive control (Perkin-Elmer, Waltham, MA).

RESULTS

Retinol Appears to Bind p110α

As can be seen in Fig. 11, retinol appears to bind p110α given the scintillation readings of 24466 and 438641 counts per minute (cpm) when 200 nM and 400 nM of ^{3}[H] retinol alone was added to immunoprecipitated p110α. In the presence of 20 and 40 μM unlabeled retinol to the cpm detected were 20018 and 19001, respectively. This indicates that the l^{3}[H] retinol was displaced by unlabeled retinol and that binding was specific. The retinol groups behaved similarly to the PI positive control. Specifically when [^{3}H]-PI was added alone, 400 nM of [^{3}H]-PI resulted in 1167 cpm ; while 200 nM of [^{3}H]-PI resulted in 1075 cpm The addition of 100-fold excess cold PI lowered the cpm to 884 cpm regardless of unlableled PI concentration. These data indicate that retinol binds to p110α, confirming the computer modeling work.

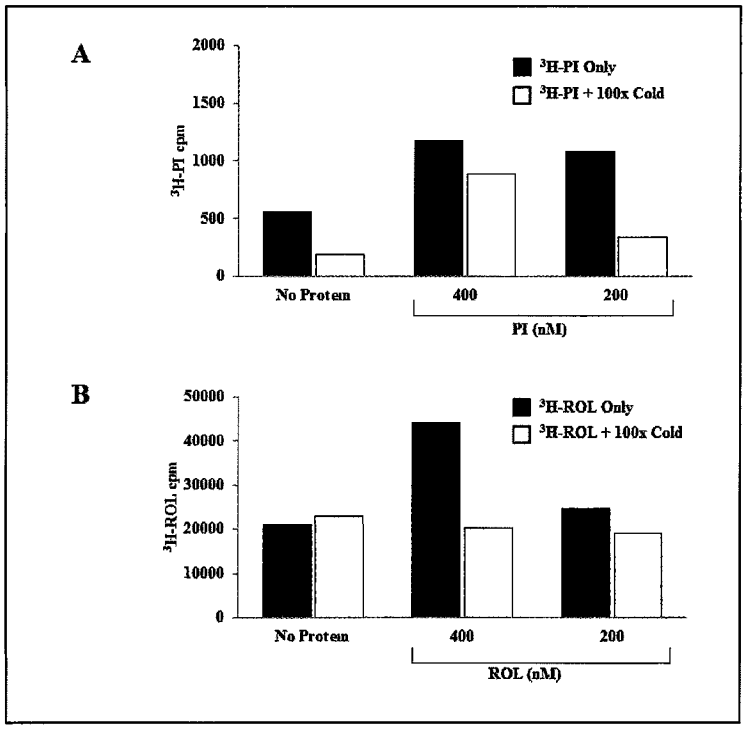

Fig 11 Total and Specific Binding to p110α. Tritiatied A) PI or B) retinol was added at either 0, 200, or 400 nM concentrations to purified p110α with or without a 100-fold molar excess of unlabeled substrate. Radioactivity was measured by scintillation counter reported as counts per minute (CPM).

DISCUSSION

The p110 subunit of PI3K contains several domains responsible for interaction with a variety of molecules which may serve to stimulate or inhibit PI3K activity (domains shown previously in Fig. 9). It is the binding of PI to the catalytic domain of p110α which allows for the conversion of PIP2 to PIP3 and subsequent propagation of the signaling cascade, which ultimately reaches the transcriptional machinery. Wortmannin inhibits p110 by binding to this region and blocking the binding of ATP, which contributes the phosphate moiety (Ihle and Powis 2010). Because retinol has been shown to inhibit the activity of PI3K (Griffin and Lane, manuscript in preparation), displays similar electrostatic characteristics to the PI3K inhibitor wortmannin (Park et al. 2008; Fig. 8), and appears to insert in the ATP binding site of p110α, our objective was to determine if retinol binds to p110α. Our data indicate that retinol does indeed bind to p110α and the amount of labeled retinol decreases when competition is introduced by non-labeled retinol, providing evidence that this binding is specific. Future studies in our laboratory will utilize [^{32}P]-ATP and unlabeled ATP and PI with the aim of revealing (through competitive binding) the exact region where retinol-p110α interaction takes place.

ACKNOWLEDGEMENTS

This research was sponsored by a grant from the American Institute for Cancer Research to Dr. Michelle Lane.

III RETINOL-INDUCED INHIBITION OF PI3K SIGNALING BYPASSES PDK-1 TO DECREASE TRANSCRIPTIONAL ACTIVATION BY NFκB AND AP-1.

ABSTRACT

Retinol has the ability to modulate cell invasion and metastasis in colorectal cancer by decreasing expression of MMP-9 as a result of PI3K signaling inhibition. This study aimed to uncover additional relationships between retinol and enzymes within this cascade, specifically targeting the master kinase 3-phosphoinositide-dependent protein kinase (PDK1), and the transcription factors nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) and activator protein-1 (AP-1). To determine if PDK1 activity was affected by retinol HCT-116 and SW620 human cancer cells were treated with and without 10 μM retinol for periods ranging from 30 min to 24 h. Western blot analysis was used to examine phospho-PDK1, which was normalized to total PDK levels. We found that retinol did not affect PDK1 activity in either cell point at any time point examined. In addition, HCT-116 and SW620 cells were transiently transfected with

reporter constructs containing the AP-1 and NFκB promoter regions upstream of luciferase to determine if retinol mediates transcription via these factors. Cells transfected with the AP-1 luciferase reporter construct were treated with either 20 ng/ml phorbol myristate acetate (PMA), 10 μM retinol, a combination of PMA and retinol, or vehicle (control). Cells transfected with the NFκB luciferase reporter construct were treated with either 20 ng/ml tumor necrosis factor alpha (TNFα), 10 μM retinol, a combination of TNFα and retinol, or vehicle (control). Though retinol appeared to slightly temper AP-1 transcriptional activity in cells treated with PMA, this effect did not display statistical significance. Retinol did not exert any effect in cells treated with NFκB. According to these outcomes, the proteins PDK-1, AP-1, and NFκB do not appear to be implicated in retinol's ability to regulate MMP-9 transcription and cell invasion in CRC.

INTRODUCTION

Our laboratory previously observed that retinol inhibits metastasis initially by inhibiting PI3K (Park et al. 2008) and ultimately by decreasing mRNA, protein, and activity of the metastatic-enhancer MMP-9 (Park et al. 2007). We also observed that retinol inhibits the activity of Akt, and either PI3K or Akt, when rendered constituently active, is sufficient to prevent the downstream effects of retinol. In addition, retinol decreases β-catenin protein levels, but over-expression of this protein does not impair the ability of retinol to decrease cell invasion (Griffin and Lane, manuscript in preparation). These findings indicate that, downstream of PI3K, Akt also modulates the ability of retinol to alter protein levels and activity within the nucleus. In contrast, retinol's ability to decrease cell invasion is independent of β-catenin, indicating that other intermediates

within the pathway are responsible for facilitating this effect. This chapter offers insight into alternate signaling components downstream of PI3K and examines whether the 3-phosphoinositide-dependent protein kinase (PDK1), and transcription mediated by nuclear factor κ-light-chain-enhancer of activated B cells (NFκB), and activator protein-1 (AP-1) are inhibited following retinol treatment.

PDK-1

PDK1 is a master kinase which functions directly downstream of PI3K and serves as a major regulator of the large "AGC" kinase family. AGC kinases are cytosolic enzymes regulated by second messenger molecules (the family is named after well-known members protein kinase A (PKA), protein kinase G (PKG), and protein kinase C (PKC), and includes the major effector of PDK1, protein kinase B/Akt. PDK1 is recruited to the cell membrane by the second messenger phospholipids PI(3,4)-bisphosphate (PIP2), to which it binds with low affinity, or PI(3,4,5)-triphosphate (PIP3) for which it has a much higher affinity (for review, see Storz and Toker 2002). PDK1 must first dock with PIP2 or PIP3 before exerting catalytic effects. Formation of these lipids recruits PDK1 to the cell membrane where interaction takes place via the Pleckstrin homology (PH) domain located C-terminally between residues 459 and 550 of PDK1. PI3K phosphorylation creates PIP2 and PIP3, which implicates PI3K as an indirect modulator of PDK1 membrane docking and kinase activity. In addition, several phosphorylation sites have been identified on PDK1 which are thought to serve a regulatory role, though there is disagreement concerning the consequence of most of these sites, as some appear to serve no purpose at all. A consensus has been reached concerning one phosphorylation event at Ser241, the only critical site where

phosphorylation is essential to PDK1 activity (Riojas et al. 2006). Until recently, disagreement also abounded regarding the mechanism and regulation of critical PDK1 phosphorylation. Now it seems most accept that phosphorylation at Ser241 is the product of autophosphorylation, rendering PDK1 constituently active. It is purported that Ser241 is resistant to dephosphorylation by phosphatases due to its inaccessible location within the protein structure. However, some dissident regarding this theory is still voiced, for example by Chen et al. who claim to have shown that PDK1 phosphorylation and subsequent activation is accomplished by insulin signaling (2001).

Substrates of PDK1 contain a hydrophobic motif commonly called a "PIF "pocket (for "PDK1-interacting fragment") where the enzyme docks to propagate the transmission of growth signals necessary for normal development (Balendran et al. 1999; Frodin et al. 2000). The role of PDK1 in development is exemplified by studies showing that mice lacking PDK1 die during early embryonic development (Mora et al. 2004) and that PDK1 deficient mice display a 40% decrease in body mass, mild glucose intolerance, and most notably, resistance to cancer induced by upregulation of the PI3K pathway (Frodin et al. 2002). To our knowledge, no study to date has looked at the effects of retinol on PDK1.

NFκB

NFκB is a rapid-acting transcription factor that induces the transcription of several anti-apoptotic proteins while suppressing transcription of pro-apoptotic proteins. For this reason, NFκB is a well-accepted inhibitor of apoptosis (Lee et al. 2007), though some evidence suggests a pro-apototic role for NFκB under certain conditions (Zanotto-Filho et al. 2009). In addition to these effects, NFκB also regulates the expression of

MMP-9 and other metastatic proteins. Blocking NFκB activity with super-repressor IκB inhibits MMP-9 expression, cell invasion, and metastasis in several cancers including colorectal cancer (Huang et al. 2001; Seo et al. 2009; Lu et al. 2010; Park et al. 2009; Schwab 2000; Yang et al. 2010). Additionally, higher levels of active NFκB were observed in tissue cultures of metastatic human head and neck squamous cell carcinoma versus non-metastatic samples, and increased NFκB activity was shown to facilitate the metastasis of human head and neck squamous cell carcinoma *in vitro* (Yan et al. 2010). Several MMP genes contain binding sites for NFκB within their promoter regions, including the gene coding for MMP-9 protein (MMP9). Furthermore, activation of NFκB is required to induce transcription of these genes (Eck et al. 2009). ATRA inhibits transcription of MMP genes, which is an effect likely mediated by antagonistic cross-coupling of transcription factors such as NFκB with RARs (Na et al. 1999). The cell lines used in this work are ATRA-resistant and do not express RAR (Lee et al. 2000; Leede et al. 1993).

AP-1

AP-1 may be active in a number signaling pathways to transcriptionally regulate several cellular processes. The outcomes of AP-1 activation are highly varied and sometimes contradictory, depending upon which pathway is responsible for its induction. When activated by MEK/ERK signaling, AP-1 drives the transcription of several inflammatory and metastatic proteins, including MMP-9, in several cancers (Weng et al. 2008; Inoue et al. 2007). Retinoids have long been known to affect AP-1-mediated gene transcription, and it is the suppression of AP-1 activity, in a manner dependent upon nuclear interactions involving retinoid ligands and DNA response elements, that

reportedly causes the anti-proliferative effects observed following retinoid treatment. This mechanism has been demonstrated using ATRA (Wu et al. 2002) as well as select synthetic retinoids (Chen et al. 1995). Because AP-1 also drives apoptosis in response to oxidative stress, concerns have been raised that retinoids may also inhibit apoptosis in certain conditions. Though some studies have reported this finding following treatment with ATRA (Kitamura et al. 2002; Zanotto-Filho et al. 2009) and 9-cis-retinoic acid (Bissonnette et al. 1995; Szondy et al. 1998), this effect is often only seen when these compounds are used at concentrations not normally reached physiologically.

Despite the abundance of data collected concerning the ability of retinoids to mediate AP-1 action and cancer metastasis, studies generally focus on forms of retinoic acid and effects that are dependent upon activation of nuclear retinoic acid receptors (RARs). To our knowledge, retinol in unaltered form has not been tested for the ability to regulate AP-1. The objectives of the current study were to determine how retinol affects proteins downstream of PI3K but upstream of MMP-9. Using two human ATRA-resistant colon cancer cells lines, western blot analysis was used to examine the effect of retinol on active PDK-1 profiles. Reporter constructs, containing the promoter regions of NFκB and AP-1 upstream of luciferase were used to determine if retinol altered NFκB or AP-1 mediated gene transcription.

MATERIALS AND METHODS

Tissue Culture

HCT-116 human colorectal carcinoma cells and SW620 human colorectal adenoma cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) in a humidified atmosphere at 37°C with 5% CO_2. The culture medium was supplemented with 10% fetal bovine serum (FBS) and antibiotics (1X penicillin/streptomycin). Prior to treatments, cells were washed with Mg^{2+}/Ca^{2+}-free phosphate buffered saline (PBS) and serum starved for 24 h in DMEM lacking supplements. The following day, media was replaced with DMEM containing FBS and antibiotics.

Western Blot Analysis

HCT-116 and SW620 human colon cancer were plated at a density of 1 x 10^6 cells per 100 mm dish and treated the following day following 24 h of serum starvation. Cells were treated with 0 or 10 μM retinol (vehicle control), 100 nM insulin, or a combination of 10 μM retinol and 100 nM insulin. Retinol treatments were performed under subdued light. Serum starvation was intended to lower phospho-PDK levels. Insulin was intended to provide a positive control by inducing PDK1 phosphorylation and catalytic activity, as was reported in 3T3 cells and rat adipose tissue following stimulation with insulin (Chen et al. 2001).

Cells were collected and lysed in a lysis buffer containing both protease and phosphatase inhibitors (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM b-glycerophosphate, 1mM sodium orthovanadate, 1 ug/ml, and 1 mM PMSF) after

treatment for 30 min, 60 min or 24 h. Lysate protein concentrations were quantified using the BioRad DC protein assay kit (Hercules, CA), after which protein (50 μg) was electrophoresed through 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Membranes were blocked with 5% bovine serum albumin (BSA) in TBST (Tris-buffered saline: 10 mM Tris (pH 8) and 150 mM NaCl; plus 0.1% Tween-20) for 1 h at room temperature with gentle shaking followed by incubation with phosphorylated PDK1 (Cell Signaling Technology, catalogue #3061, Danvers, MA) primary antibody at a 1:1000 dilution in 4°C overnight with gentle shaking. The following day membranes were incubated with corresponding secondary antibody (Santa Cruz Biotechnology, catalogue #sc-2004, Santa Cruz, CA) at a 1:2000 dilution for 1 h at room temperature with gentle shaking. After probing for phosphorylated protein, membranes were stripped and the process repeated using a primary antibody that detected phosphorylated and unphosphorylated forms of PDK1 (Cell Signaling Technology, catalogue #3062, Danvers, MA). Membranes were then stripped again and probed for β-actin by blocking with 5% milk in place of BSA and incubating with primary antibody (Sigma Aldrich, catalogue #A1978, St. Louis, MO) for only 1 h at a 1:2000 dilution before proceeding to secondary antibody (Santa Cruz Biotechnoloy, catalogue #sc-358914, Santa Cruz, CA). Immunoreactivity was detected using the Pierce Horseradish Peroxidase Super Signal West Pico Chemiluminescent Substrate kit (catalogue # 34087, Rockford, IL) and resulting bands were captured using Fotodyne PC Image (Fotodyne, Inc., Hartland, WI). Bands displayed within the appropriate molecular weight range were quantified using ImageQuant TL software (GE Healthcare Life Sciences, Piscataway, NJ).

Transient Transfection and Luciferase Assays

HCT-116 and SW620 cells were plated in 6-well plates at a density of $1x10^5$ cells per well and incubated overnight in DMEM supplemented with 10% FBS and antibiotics. The following day, cells were transfected with1 μg of the luciferase reporter constructs AP-1 (pAP1(2)-Luc, Affymetrix, catalogue #LR0003, Santa Clara, CA) or NFκB (pNFκB-luc, Affymetrix, catalogue #LR0051, Santa Clara, CA), or the TA-luc empty vector control (Affymetrix, catalogue #LR0000, Santa Clara, CA), as well as 1 μg of pSV-β-galactosidase (a generous gift from Dr. Dianne Soprano, Temple University, Philadelphia, PA) in antibiotic-free DMEM supplemented with 10% FBS using Lipofectamine 2000 reagent as directed by manufacturer's protocol (Invitrogen, Carlsbad, CA). Following a 3 h incubation at 37°C, media was replaced with media containing antibiotics and cells were given time to recover from transfection. The following day, cells were serum starved for 24 h as described previously. Following serum starvation, cells transfected with AP-1 reporter were given complete media containing either 20 ng/ml phorbol myristate acetate (PMA), 10 μM retinol, a combination of PMA and retinol, or vehicle (control). Cells transfected with the NFκB luciferase reporter were treated with either 20 ng/ml tumor necrosis factor alpha (TNFα), 10 μM retinol, a combination of TNFα and retinol, or vehicle (control). PMA and TNFα are known to stimulate transcription via AP-1 and NFκB responsive elements respectivity (Khalaf et al. 2010; Ohmori and Hamilton 1995) and were used as positive controls. Cells were harvested 24 h after treatment and luciferase and β-galactosidase activity assessed in the lysates. Each experiment was repeated three or four times. Cells were lysed by adding 300 μL Reporter Lysis Buffer (Promega, catalogue # E1531, Madison,

WI) per well and incubating for 15 min at room temperature with gentle rocking. Manual lifting of cells followed to collect any cells still adhered to wells. The lysate/buffer solution was aspirated and kept on ice or stored at -80°C in 1.5 ml eppendorf tubes. Luciferase activity was measured following one freeze-thaw cycle using the Promega Luciferase Assay System (catalogue #E4030, Madison, WI) per manufacturer instructions. β-Galactosidase activity was used to correct for transfection efficiency (β-Galactosidase Enzyme Assay System, Promega, Madison, WI).

Statistical Analysis

Results are expressed as % vehicle control and are shown as the mean ± SEM of three (western blot analysis) or four (luciferase assays) biological repeats. Significant differences when compared to control were determined using a Mann-Whitney U test performed in SPSS version 19 (IBM, Chicago, IL). *P*-values less than 0.05 were considered significant.

RESULTS

Retinol's Ability to Decrease Colon cancer Metastasis via PI3K Signaling is Independent of PDK-1.

Retinol has the ability to modulate cell invasion and metastasis in colorectal cancer by decreasing expression of MMP-9 as a result of PI3K signaling inhibition. Downstream of PI3K, Akt and β-catenin are also subject to inhibition by retinol, but inhibition of β-catenin alone fails to result in lower cell invasion and MMP-9 activity (Griffin and Lane, manuscript in preparation). The intermediates responsible for propagating this regulatory impulse, from origin at PI3K upon interaction with retinol, to the ultimate site of retinol regulation within the nucleus, have yet to be deciphered. This study aimed to uncover additional relationships between retinol and enzymes within this

cascade, beginning with the master kinase PDK-1. To measure PDK-1 activity, we performed western blot analyses using a primary antibody specific for phosphorylation at the critical residue, Ser-241 (Casamayor et al. 1999). Our findings indicate that cells treated with retinol, insulin, or a combination of retinol and insulin exhibited no changes in phosphorylated PDK-1 as a proportion of total protein levels when compared to vehicle-treated control cells at any time point in either cell line (Figs 12 and 13). The clear lack of change in activated PDK-1 levels observed within insulin-treated cells indicate that PDK-1 is indeed autonomous. This is consistent with the view that PDK-1 is a constituently active kinase and dependent upon autophosphorylation at this crucial residue that is unresponsive to changes in insulin signaling and not influenced by treatment with retinol (Cheng et al. 2011; Filippa et al. 2000). Importantly, retinol did not alter the phosphorylation level of PDK-1 in either cell line at any time point examined. Thus, PDK-1 is not an intermediate in the pathway linking PI3K to MMP-9 and metastasis.

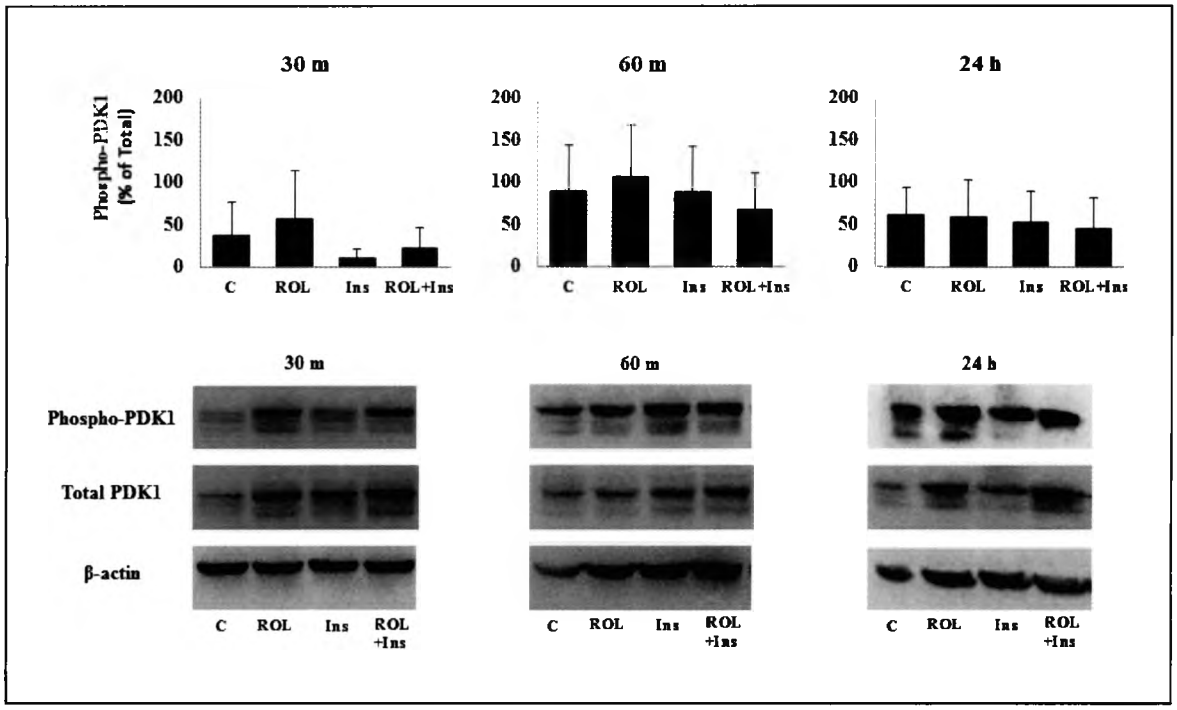

Fig. 12 Phosphorylated Levels of PDK-1 with Various Treatments in HCT-116 Cells. Graphs depict phosphorylated levels of PDK-1 expressed as percent of total PDK-1 protein for cells treated with vehicle control (C), 10 μM retinol (ROL), 20 ng/ml insulin (Ins), or a combination of retinol and insulin (ROL+Ins). Western blots display Ser241 phosphorylation of PDK-1, total levels of PDK-1, and β-actin in corresponding groups.

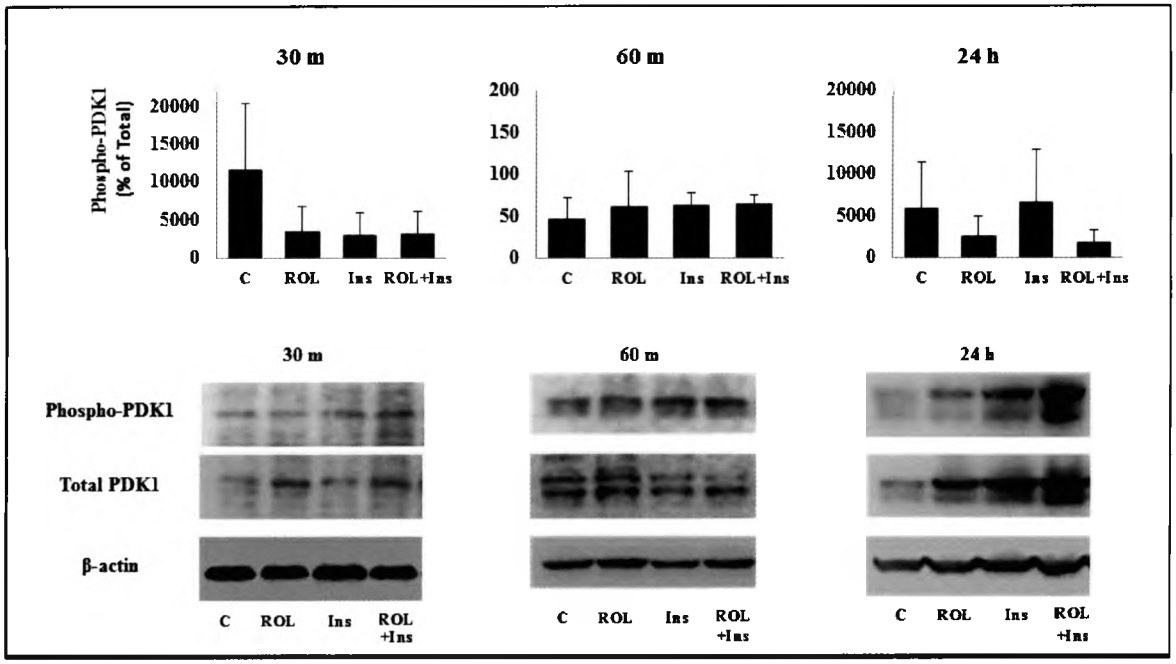

Fig. 13 Phosphorylated Levels of PDK-1 with Various Treatments in SW620 Cells. Graphs depict phosphorylated levels of PDK-1 expressed as percent of total PDK-1 protein for cells treated with vehicle control (C), 10 μM retinol (ROL), 100 nM insulin (Ins), or a combination of retinol and insulin (ROL+Ins). Western blots display Ser241 phosphorylation of PDK-1, total levels of PDK-1, and β-actin in corresponding groups.

Retinol does not exert a significant effect on AP-1 and NFκB transcriptional regulation.

The promoter region of the MMP-9 gene contains both AP-1 and NFκB responsive elements (Li et al. 2010). To determine if retinol could inhibit AP-1 or NFκB-mediated gene transcription, HCT-116 and SW620 cells were transfected with luciferase reporter constructs containing AP-1 or NFκB responsive elements as well as a luciferase construct lacking these elements. HCT-116 and SW620 cells transfected with the AP-1 reporter construct responded to treatment with PMA, an AP-1 activator, with an expected increase in luciferase activity. In HCT-116 cells treated for 24 h, PMA induced a spike in luciferase activity reaching 290 ± 72% that of control ($P = 0.02$) (Fig. 14A). Treatment with both PMA and retinol tended to reduce luciferase activity to 125 ± 49% of control ($P = 0.16$ vs PMA alone). In SW620 cells, luciferase activity increased to 439 ± 152% control ($P = 0.02$) following 24 h of treatment with PMA. Treatment with PMA and retinol reduced luciferase activity to 234 ± 88% of control, but this decrease was not significant when compared to PMA alone ($P = 0.39$; Fig. 14B). These data indicate that retinol may curb, to some extent, transcription via AP-1 responsive elements in HCT-116 cells. We theorize that modulation of AP-1 may account, at least in part, for the ability of retinol to decrease MMP-9 mRNA levels in these cell lines.

Stimulation with TNFα caused increased luciferase activity in HCT-116 (417 ± 142% of control; $P = 0.04$) and SW620 cells (584 ± 137% of control; $P = 0.04$), as expected (Fig. 15A) when transfected with the NFκB reporter construct. HCT-116 cells showed a slight decrease in the luciferase activity driven by the NFκB responsive promoter following treatment with a combination of retinol and TNFα to 243 ± 92% control; however, this decrease was not statistically significant ($P = 0.28$ vs TNFα).

Treatment of SW620 cells transfected with the NFκB reporter construct with a combination of retinol and TNFα did not result in decreased luciferase activity when compared to TNFα alone (Fig. 15B). The small variations in luciferase activity observed between groups treated with TNFα alone versus those treated with TNFα plus retinol indicate that retinol is probably not having on effect on NFκB-mediated transcription.

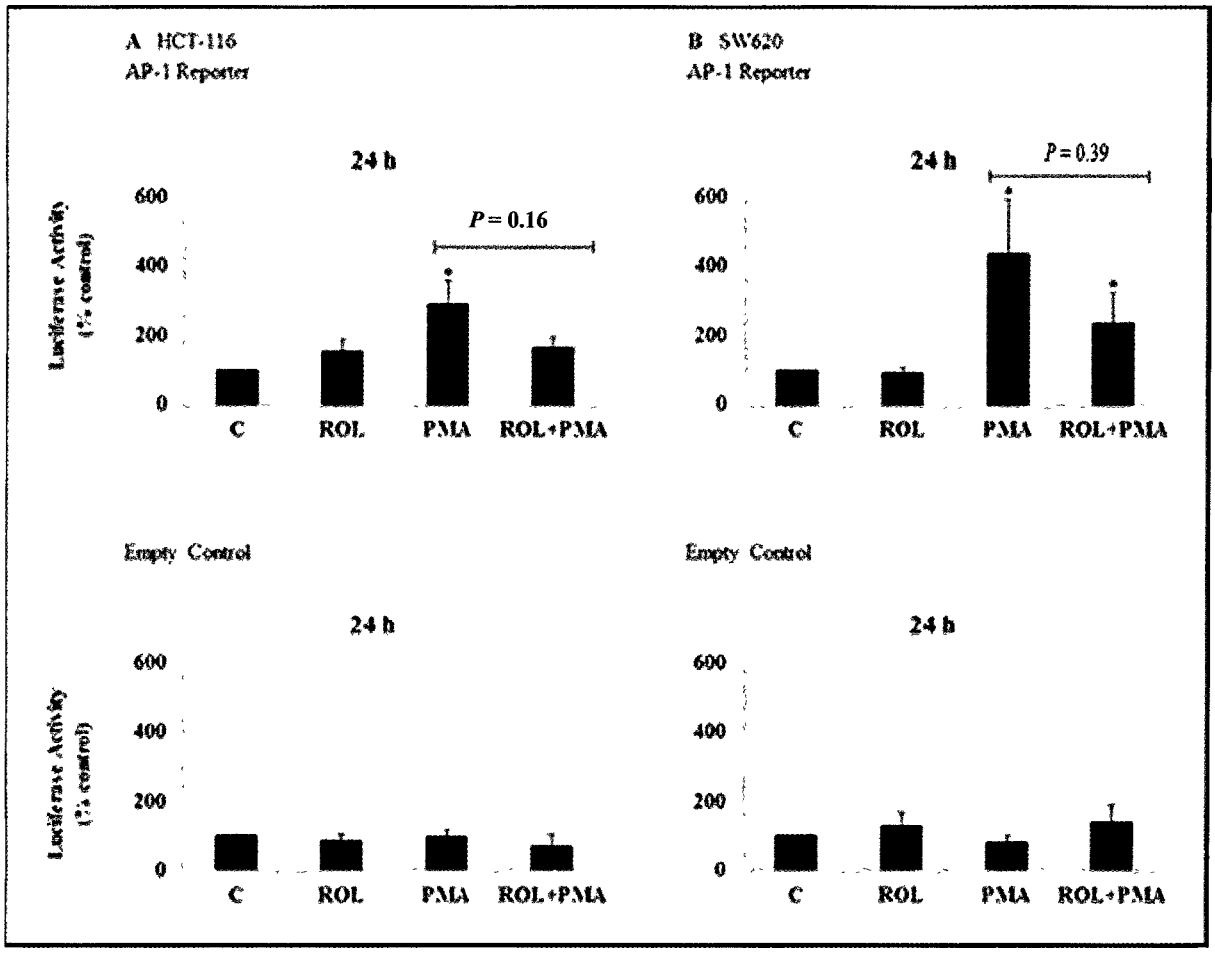

Fig. 14 AP-1 Reporter-driven Luciferase Activity. (A) HCT-116 and (B) SW620 cells transfected with AP-1 reporter construct or empty control vector were treated with vehicle control (C), 10 μM retinol (ROL), 20 ng/ml of the AP-1 stimulator phorbol myristate acetate (PMA), or a combination of PMA and retinol for 24 h. All data are reported as mean ± SE for n=4. *Significantly different from control; $P < 0.05$.

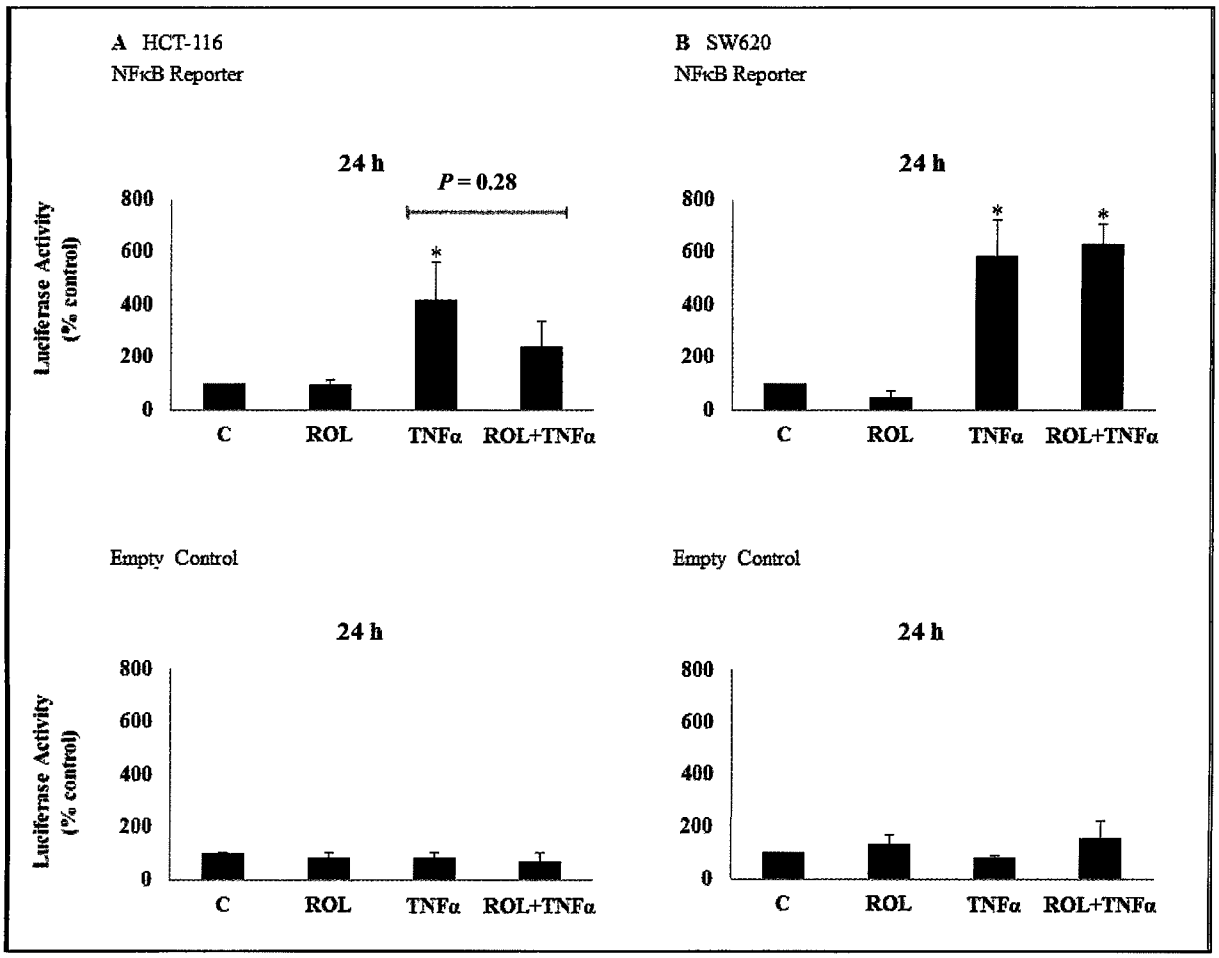

Fig. 15 NFκB Reporter-driven Luciferase Activity. (A) HCT-116 and (B) SW620 cells transfected with AP-1 reporter construct or empty control vector were treated with vehicle control (C), 10 μM retinol (ROL), 20 ng/ml of the NFκB stimulator tumor necrosis factor α (TNFα), or a combination of TNFα and retinol for 24 h. All data are reported as mean ± SE for n=4. *Significantly different from control; $P < 0.05$.

DISCUSSION

Previous work in our laboratory found that retinol decreased PI3K activity and cell invasion (Griffin and Lane, manuscript in preparation). In addition, retinol decreases the mRNA, protein, and activity levels of the pro-metastatic protein MMP-9 (Park et al. 2007). The ability of retinol to repress cell invasion and metastasis in colorectal cancer is contingent upon its ability to decrease the expression of MMP-9 within the nucleus, which is accomplished by inhibition of PI3K and continuous modulation along the entire length of the PI3K signaling pathway. Downstream of PI3K, Akt and β-catenin are also subject to inhibition by retinol, but β-catenin fails to extend this effect further along the signaling pathway. Specifically, the decrease in □-catenin levels following retinol treatment is not sufficient to decrease cell invasion or MMP-9 levels alone (Griffin and Lane, manuscript in preparation). The intermediates responsible for propagating this regulatory impulse, from origin at PI3K upon interaction with retinol, to the ultimate site of retinol regulation within the nucleus, have yet to be deciphered. The purpose of this study was to further understanding of retinol's impact within the PI3K cascade. Specifically, we aimed to determine if treatment with retinol modulate PDK1 activity and by AP-1 and NFκB -mediated gene transcription.

In the currently study we expected retinol's inhibitory effect on PI3K to be reflected throughout the PI3K signaling pathway with down-regulation of downstream protein activity and ultimate AP-1 and NFκB-driven protein expression. Specifically, we expected diminished phosphorylated-PDK-1 as well as decreased AP-1 and NFκB -mediated gene transcription following retinol treatment. We treated two human colon cancer cells with 10 μM retinol with and without insulin to examine the effect of retinol

on PDK1 phosphorylation. However, there is a good amount of conflict concerning whether or not PDK1 is actually regulated by components of the PI3K signaling pathway (see Mora et al. 2004 for review). Our results indicate that PDK1 in fact does not appear to alter activity in response to insulin signaling (Figs. 8 and 9). Though no other studies have looked at the relationship between retinol and PDK1, Hughes et al. (2006) did show that the ability of retinoids (ATRA and 9-*cis*-retinoic acid) to enhance the production of steroid sulfatase in leukemia cells is dependent upon PDK1 and other proteins within the MEK/ERK pathway. In future studies, the inclusion of other growth factors as well as second messenger molecules such as PIP2 and PIP3 will help shed light on what influences may be exerted over PDK1 activity. More importantly regarding further query into the retinol question, other intermediates linking PI3K, Akt, and MMP-9 must be evaluated in order to uncover which continuous pathway might subject to retinol inhibition and how inhibition is being accomplished.

In order to see if retinol transcriptionally regulated the production of MMP-9 through modulation of either AP-1 or NFκB, cells were transfected with plasmid vectors containing the appropriate responsive elements upstream of the luciferase (Luc) gene. Our investigation showed no significant effect of retinol treatment on transcriptional induction by these proteins; however, retinol did tend to reduce the ability of PMA to induce transcription via AP-1 particularly in HCT-116 cells (Fig. 10). Other retinoids have been shown to affect transcription of AP-1 and NFκB products, though not necessarily through regulation of these transcription factors (Farhana et al. 2010; Chen et al. 1995; Wu et al. 2002). In addition, these alterations were mediated by RARs which our cell lines lack. Controversy exists here as well, as some researchers claim retinoids

inhibit the ability of cells to undergo apoptosis in response to cellular damage by inhibiting AP-1-mediated apoptotic processes. One such argument is found in a study focusing on oxidative stress, Zanotto-Filho et al.. (2009) found that NFκB mediates cellular resistance to the pro-oxidant effects of vitamin A by inhibiting the accumulation of reactive species and the redox-dependent activation of AP-1. For our investigation, it is important to note that retinol does not induce apoptosis in ATRA-resistant human CRC cells (Park et al. 2005). Also, the concentrations of retinol used in our laboratory are physiologically relevant and much lower than that required to induce retinol-mediated oxidation. In conclusion, the activity of PDK1 is not reduced by retinol. NFκB activity appears to be unaltered by retinol in stimulated SW620 cells, though retinol treatment appears slightly hamper AP-1 and possibly NFκB-mediated gene transcription in stimulated HCT-116 cells. However, the small size of this effect may amount to this observed inhibition lacking biological relevance. Further research may reveal if this mediation contributes in part to retinol's decrease of invasion and metastasis in HCT-116 cells. To determine this, the effect must be confirmed in a model examining endogenous AP-1 and NFκB-mediated gene expression. Some other proteins of note are suggested in Chapter 4 as future areas of focus.

ACKNOWLEDGEMENTS

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IV FUTURE DIRECTIONS

INTRODUCTION

Beyond the narrow scope of this work, a broader view of the PI3K signaling pathway reveals a complex network formed by the convergence of multiple protein cascades. Within each segment, more key regulatory proteins emerge as potential points of dysfunction and/or possible propagators of retinol mediation. Examples of some such important kinases include phosphatase and tensin homolog (PTEN), extracellular signal related kinase 1 and 2 (ERK), and mammalian target of rapamycin (mTOR). Further research into the interactions governing this immense pathway, and how those interactions change in the case of cancer or following retinol treatment, is necessary to further our understanding of retinol's chemopreventative or chemotherapeutic potential. The proteins discussed in this chapter suggest the most obvious sites to direct the focus of future research.

POTENTIAL RESEARCH TARGETS

PTEN

Phosphatase and tensin homolog (PTEN) is a phosphoinositide-3-phosphatase that acts as a tumor suppressor and a regulator of cellular metabolism (Wishart and Dixon 2002). Stimulation of PI3K causes the production of PIP3, which recruits Akt to the cell membrane where it is phosphorylated by PDK1. PTEN functions in opposition of PI3K to catalyze the dephosphorylation of membrane lipids, specifically the conversion of PIP3 to PIP2, effectively down-regulating Akt (Yin and Shen 2008). PTEN is one of the most commonly lost tumor suppressors in human cancer. As cells transform, mutations accumulate in the PTEN gene eventually leading to the loss of enzyme function, resulting

in dysregulation of apoptotic functions and enhanced cell survival. Furthermore, inactivation of PTEN has been implicated in the development of chemotherapy resistance (Steelman et al. 2008). Frequent genetic inactivation of PTEN occurs in glioblastoma, endometrial cancer, prostate cancer, and reduced expression is found in many other tumor types such as lung and breast cancer (Yuan and Cantley 2008). Several reactive aldehydes and ketones have been found to covalently modify and inactivate PTEN resulting in subsequent activation of Akt (PKB) kinase, phosphorylation of Akt substrates, increased nuclear β-catenin signaling, and increased cell proliferation (Covey et al. 2010). Treatment with ATRA in combination with IFN-γ controlled the growth of both PTEN-proficient (in which PTEN was up-regulated after treatment) and PTEN-deficient glioblastoma cells by inducing differentiation and apoptosis (Zhang et al. 2008). One group demonstrated that retinoids target both PI3K and PTEN through transcriptional processes involving RAR/RXR heterodimers (Bastien et al. 2004). Given the role PTEN plays in tumor suppression, investigation into a possible retinol-PTEN interaction is warranted. Future undertakings should answer whether or not retinol has the ability to up-regulate PTEN function to further inhibit PI3K signaling.

ERK

The complex known as extracellular signal-regulated kinases 1 and 2, or just extracellular-regulated kinase (ERK) exists within the Raf/MEK/ERK cascade which regulates the transcription of genes related to proliferation and cell. Activation of this sub-pathway is propagated by PI3K signaling at multiple points, which were demonstrated and quantified by Wang and colleagues (2009). The current breadth of knowledge concerning MEK/ERK signaling is relatively new and quickly expanding. At

present, multiple transcription factors are known to be induced by activated ERK including c-Myc, Ets, CREB, c-Jun, c-Fos, and most notably NFκB. In addition to transcriptional regulation, this pathway is also responsible for post-translational regulation (phosphorylation) of apoptotic regulatory enzymes (for a review, see McCubrey et al. 2006). Abnormal activation of this signaling has been observed in human cancers as either secondary to PI3K, Akt, or PTEN mutations (Lee et al. 2004; Steelman et al. 2009) or from mutations within the Raf/MEK/ERK pathway itself— the latter of which further complicates matters by resulting in decreased PTEN expression. This pathway has been implicated in drug resistance via several mechanisms compiled by McCubrey and colleagues in an additional review (2011). Up-regulated Raf/MEK/ERK signaling induced by treatment with the chemotherapy drugs Doxorubicin and Docetaxel may give rise to drug resistance in daughter cells (McCubrey et al. 2006), and artificial activation of Raf has been shown to induce resistance to the chemotherapeutic agents Doxorubicin and Paclitaxel in breast cancer cells (Garnett and Marais 2004). Dysregulation of this pathway also reduces the expression of proteins that repair DNA, further contributing to resistant cancer (for review, see McCubrey et al. 2011). Mutations in the gene encoding upstream kinases (the RAS/RAF gene) are seen in over 25% of all tumors. In colorectal cancer specifically, the number jumps to greater than 50% (Nandan and Yang 2011).

Because of observations linking Raf/MEK/ERK functioning to cancer, this pathway has gained popularity as a target for new cancer modalities. In the absence of drug resistance, inhibitors of these kinases and some downstream products have seen success in clinical trials (Wang et al. 2007). Given the relationship between

Raf/MEK/ERK and PI3K signaling, it is reasonable to consider that retinol may inhibit the spread of colon cancer at least partially via modulation of Raf/MEK/ERK. A reasonable place to start testing this theory would be with ERK. Due to the downstream location of this kinase, examination of ERK would be sufficient to determine whether or not retinol modulation is occurring in the pathway at large. More importantly, ERK directly activates NFκB which has been shown in the previous chapter to be a likely conduit of retinol inhibition. The observed alteration in NFκB function may be illustrative of a link between ERK and decreased MMP-9 expression resulting in decreased metastasis. Proteins occurring farther upstream, particularly those found mutated in cancer (Ras/Raf) are also enticing targets for study. Nevertheless, progression directly upstream of NFκB— to ERK— is the most reasonable next step in the interest of uncovering continuity in the retinol-dependent inhibition of metastasis.

mTOR

Mammalian target of rapamycin (mTOR) is a key component of two enzyme complexes: mammalian target of rapamycin complex 1 and 2 (mTORC1 and mTORC2) occurring within the mTOR pathway. The markedly different locations at which each of these complexes interacts with the PI3K cascade makes mTOR a key regulator in both upstream and downstream PI3K signaling. For example, activated Akt can activate mTOR downstream, which may then contribute either negative feedback to inactivating Akt (via mTORC1 action on p70S6K) or positive feedback further activating Akt (via a critical phosphorylation provided by mTORC2) (reviewed by McCubrey et al. 2011). While PDK1 is responsible for a crucial phosphorylation at Thr308 of Akt, mTORC2 is now known to be the enzyme responsible for a second critical phosphorylation event at

Ser473 (Martelli et al. 2009; Sarbassov et al. 2005) (mTORC2 was formerly referred to as PDK2 in this capacity). More is known about the mTORC1 complex, which largely promotes anabolic processes contributing to growth and proliferation. Growth factors stimulate mTORC1 through Akt action (PI3K signaling) as well as ERK action propagated through Ras/MEK/ERK signaling. Not surprisingly, mTORC1 has been shown to be active during pro-cancer processes such as tumor formation and angiogenesis, and dysregulation of the mTOR pathway has been implicated in cancer (reviewed by McCubrey et al. 2011). Some epidemiological data points to constitutively-active mTORC1 in over 90% of primary acute myeloid leukemia (Tamburini et al. 2009). In his review of mTOR and cancer, Sabatini (2006) notes that mTORC1 is active in most "if not all" tumors. Similarly to PTEN and ERK, mTOR engages in considerable crosstalk with the PI3K pathway, serves as an important regulator at key points of signaling, and has demonstrated an abnormal level of activity in cancer. As such, mTOR is another important point to examine with respect to MMP-9 expression/metastasis and potential modulation by retinol.

Though what is known about mTOR falls short when compared to the depth of available data related to PTEN and even ERK, deciphering the role mTOR plays in cancer metastasis is of particular importance because it may uncover implications for certain population subgroups. A popular practice among the strength training and body building communities is supplementation of branched-chain amino acids (BCAAs), specifically leucine, in an attempt to maximize muscle fiber synthesis following resistance exercise. In fact, leucine-rich BCAA supplementation has been shown to upregulate mTOR signaling and protein synthesis in human muscle tissue (Dreyer et al.

2008). Increased leucine levels appear to shift protein turnover in favor of muscle growth at the translational level, an effect accomplished in part by mTOR-mediated processes [release of initiation factor 4 complex (eIF4E) and activation of the 70 kDa rpS6-kinase ($p70^{s6}K$)] (Anthony et al. 2000; Anthony et al. 2001). Leucine works synergistically with insulin to enhance PI3K signaling in muscle (Anthony et al. 2001; Layman 2002) as well as in adipocytes in the mouse obesity (db/db) model, where it has even been demonstrated to overcome PI3K/Akt inhibition by wortmannin (Hinault et al. 2004). It is reasonable to speculate that a consequence of such supplementation and artificial mTOR stimulation may be exacerbated prolific activity in cancerous cells and induced metastasis of pre-existing tumors if what is seen in muscle tissue also occurs in epithelium. As such, this population may be at higher risk of metastatic colon cancer as a result of leucine supplementation, especially if also engaging in the more extreme practice of supplementing insulin for maximal muscle gains. Much more research is needed in order to determine if leucine stimulates mTOR/PI3K signaling in epithelial cells; if leucine supplementation correlates with colon cancer incidence, metastasis, and mortality; and if this signaling is subject to inhibition by retinol.

CONCLUSION

Several signaling pathways interact with the PI3K cascade at various points to create an elaborate signaling network. The points of crosstalk between pathways provide potential avenues through which irregular signals may be propagated. Important proteins in this regard include PTEN, ERK, and mTOR. PTEN is of particular interest because its action directly counters that of PI3K, and similar to PI3K, PTEN activity is often abnormal in cancerous tissue. Furthermore, retinoids have been demonstrated to affect

PTEN expression at the nuclear level. The protein ERK provides a leg of the PI3K cascade directly upstream of NFκB. ERK goes on to propagate the Raf/MEK/ERK pathway which is also abnormal in many cancers as an effect of PI3K, Akt, or PTEN mutations. Notably, mutations within Raf/MEK/ERK itself have been shown to independently decrease PTEN expression. The mTOR complex provides another interesting point of study as it interacts with both upstream and downstream proteins within the PI3K cascade. In addition, mTOR is a key activator of this pathway in response to the popular supplemental BCAA leucine. For these reasons, it would be beneficial to expand the investigation of retinol's effects on cancer signaling outside the conventional PI3K pathway to related pathways, starting with these specific points of intersection. This knowledge will provide a more accurate portrait of retinol's potential as a preventative agent or therapy of metastatic cancer.

WORKS CITED

Anthony JC, Lang CH, Crozier SJ, Anthony TG, MacLean DA, Kimball SR, Jefferson LS. Contribution of insulin to the translational control of protein synthesis in skeletal muscle by leucine. Am J Physiol Endocrinol Metab. 2002 May;282(5):E1092-101. PubMed PMID: 11934675.

Anthony JC, Yoshizawa F, Anthony TG, Vary TC, Jefferson LS, Kimball SR. Leucine stimulates translation initiation in skeletal muscle of postabsorptive rats via a rapamycin-sensitive pathway. J Nutr. 2000 Oct;130(10):2413-9. PubMed PMID: 11015466.

Anthony TG, Anthony JC, Yoshizawa F, Kimball SR, Jefferson LS. Oral administration of leucine stimulates ribosomal protein mRNA translation but not global rates of protein synthesis in the liver of rats. J Nutr. 2001 Apr;131(4):1171-6.

Altucci L, Leibowitz M D, Ogilvie KM, de Lera AR, Gronemeyer H (2007). "RAR and RXR modulation in cancer and metabolic disease." Nat Rev Drug Discov. 6(10):793-810.

Andela VB, Rosier RN.The proteosome inhibitor MG132 attenuates retinoic acid receptor trans-activation and enhances trans-repression of nuclear factor kappaB. Potential relevance to chemo-preventive interventions with retinoids.Mol Cancer. 2004 Mar 22;3:8.PubMed [citation] PMID: 15035668, PMCID: PMC398417.

Arcaro A, Guerreiro AS.The phosphoinositide 3-kinase pathway in human cancer: genetic alterations and therapeutic implications.Curr Genomics. 2007 Aug;8(5):271-306.PubMed [citation] PMID: 19384426, PMCID: PMC2652403.

Bissonnette RP, Brunner T, Lazarchik SB, Yoo NJ, Boehm MF, Green DR, Heyman RA. 9-cis retinoic acid inhibition of activation-induced apoptosis is mediated via regulation of fas ligand and requires retinoic acid receptor and retinoid X receptor activation. Mol Cell Biol. 1995 Oct;15(10):5576-85. PubMed PMID: 7565709; PubMed Central PMCID: PMC230808.

Balendran A, Currie R, Armstrong CG, Avruch J, Alessi DR. Evidence that 3-phosphoinositide-dependent protein kinase-1 mediates phosphorylation of p70 S6 kinase in vivo at Thr-412 as well as Thr-252. J Biol Chem. 1999 Dec 24;274(52):37400-6. PubMed PMID: 10601311.

Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z, Hanahan D.Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis.Nat Cell Biol. 2000 Oct;2(10):737-44.PubMed [citation] PMID: 11025665, PMCID: PMC2852586.

Bernhard EJ, Gruber SB, Muschel RJ.Direct evidence linking expression of matrix metalloproteinase 9 (92-kDa gelatinase/collagenase) to the metastatic phenotype in transformed rat embryo cells.Proc Natl Acad Sci U S A. 1994 May 10;91(10):4293-7.PubMed [citation] PMID: 8183903, PMCID: PMC43771.

Brtko J.Retinoids, rexinoids and their cognate nuclear receptors: character and their role in chemoprevention of selected malignant diseases.Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2007 Dec;151(2):187-94. Review.PubMed [citation] PMID: 18345250.

Casamayor A, Morrice NA, Alessi DR. Phosphorylation of Ser-241 is essential for the activity of 3-phosphoinositide-dependent protein kinase-1: identification of five sites of phosphorylation in vivo. Biochem J. 1999 Sep 1;342 (Pt 2):287-92. PubMed PMID: 10455013; PubMed Central PMCID: PMC1220463.

Center MM, Jemal A, Ward E.International trends in colorectal cancer incidence rates.Cancer Epidemiol Biomarkers Prev. 2009 Jun;18(6):1688-94.PubMed [citation] PMID: 19505900.

Chen H, Nystrom FH, Dong LQ, Li Y, Song S, Liu F, Quon MJ. Insulin stimulates increased catalytic activity of phosphoinositide-dependent kinase-1 by a phosphorylation-dependent mechanism. Biochemistry. 2001 Oct 2;40(39):11851-9. PubMed PMID: 11570885.

Chen JS, Wang Q, Fu XH, Huang XH, Chen XL, Cao LQ, Chen LZ, Tan HX, Li W, Bi J, Zhang LJ.Involvement of PI3K/PTEN/AKT/mTOR pathway in invasion and metastasis in hepatocellular carcinoma: Association with MMP-9.Hepatol Res. 2009 Feb;39(2):177-86.PubMed [citation] PMID: 19208038.

Chen JY, Penco S, Ostrowski J. (1995) "RAR-specific agonist/antagonists which dissociate transactivation and API transrepression inhibit anchorage-independentcell proliferation." EMBO J., 14:1187-1197.

Cheng JC, Chou CH, Kuo ML, Hsieh CY.Radiation-enhanced hepatocellular carcinoma cell invasion with MMP-9 expression through PI3K/Akt/NF-kappaB signal transduction pathway.Oncogene. 2006 Nov 9;25(53):7009-18. Epub 2006 May 29.PubMed [citation] PMID: 16732316.

Cheng L, Locke C, Davis GW.S6 kinase localizes to the presynaptic active zone and functions with PDK1 to control synapse development.J Cell Biol. 2011 Sep 19;194(6):921-35.PubMed PMID: 21930778, PMCID: PMC3207287.

Chung AW, Radomski A, Alonso-Escolano D, Jurasz P, Stewart MW, Malinski T, Radomski MW.Platelet-leukocyte aggregation induced by PAR agonists: regulation by nitric oxide and matrix metalloproteinases.Br J Pharmacol. 2004 Dec;143(7):845-55. Epub 2004 Nov 8.PubMed [citation] PMID: 15533889, PMCID: PMC1575938.

Collins I, Workman P.New approaches to molecular cancer therapeutics.Nat Chem Biol. 2006 Dec;2(12):689-700. Review. Erratum in: Nat Chem Biol. 2007 Feb;3(2):126. PubMed [citation] PMID: 17108987.

Covey TM, Edes K, Coombs GS, Virshup DM, Fitzpatrick FA (2010). "Alkylation of the tumor suppressor PTEN activates Akt and β-catenin signaling: a mechanism linking inflammation and oxidative stress with cancer." PLoS One 5(10): e13545.

Cragg GM, Grothaus PG, Newman DJ.Impact of natural products on developing new anti-cancer agents.Chem Rev. 2009 Jul;109(7):3012-43. Review. No abstract available. PubMed [citation] PMID: 19422222.

Dillard AC, Lane MA.Retinol decreases beta-catenin protein levels in retinoic acid-resistant colon cancer cell lines.Mol Carcinog. 2007 Apr;46(4):315-29.PubMed [citation] PMID: 17219422.

Dreyer HC, Drummond MJ, Pennings B, Fujita S, Glynn EL, Chinkes DL, Dhanani S, Volpi E, Rasmussen BB. Leucine-enriched essential amino acid and carbohydrate ingestion following resistance exercise enhances mTOR signaling and protein synthesis in human muscle. Am J Physiol Endocrinol Metab. 2008 Feb;294(2):E392-400. Epub 2007 Dec 4. PubMed PMID: 18056791; PubMed Central PMCID: PMC2706121.

Dutta A, Sen T, Banerji A, Das S, Chatterjee A (2009). "Studies on Multifunctional Effect of All-Trans Retinoic Acid (ATRA) on Matrix Metalloproteinase-2 (MMP-2) and Its Regulatory Molecules in Human Breast Cancer Cells (MCF-7)." J Oncol 2009: 627840.

Eck SM, Blackburn JS, Schmucker AC, Burrage PS, Brinckerhoff CE. Matrix metalloproteinase and G protein coupled receptors: co-conspirators in the pathogenesis of autoimmune disease and cancer. J Autoimmun. 2009 Nov-Dec;33(3-4):214-21. Epub 2009 Oct 1. Review. PubMed PMID: 19800199; PubMed Central PMCID: PMC2783549.

Edwards BK, Ward E, Kohler BA, Eheman C, Zauber AG, Anderson RN, Jemal A, Schymura MJ, Lansdorp-Vogelaar I, Seeff LC, van Ballegooijen M, Goede SL, Ries LA.Annual report to the nation on the status of cancer, 1975-2006, featuring colorectal cancer trends and impact of interventions (risk factors, screening, and treatment) to reduce future rates.Cancer. 2010 Feb 1;116(3):544-73.PubMed [citation] PMID: 19998273.

Ericson K, Gan C, Cheong I, Rago C, Samuels Y, Velculescu VE, Kinzler KW, Huso DL, Vogelstein B, Papadopoulos N.Genetic inactivation of AKT1, AKT2, and PDPK1 in human colorectal cancer cells clarifies their roles in tumor growth regulation.Proc Natl Acad Sci U S A. 2010 Feb 9;107(6):2598-603. Epub 2010 Jan 20. Erratum in: Proc Natl Acad Sci U S A. 2010 Apr 6;107(14):6551. PubMed [citation] PMID: 20133737, PMCID: PMC2823889.

Farhana L, Dawson MI, Xia Z, Aboukameel A, Xu L, Liu G, Das JK, Hatfield J, Levi E, Mohammad R, Fontana JA. Adamantyl-substituted retinoid-related molecules induce apoptosis in human acute myelogenous leukemia cells. Mol Cancer Ther. 2010 Nov;9(11):2903-13. Epub 2010 Nov 9. PubMed PMID: 21062916; PubMed Central PMCID: PMC3058343.

Frödin M, Jensen CJ, Merienne K, Gammeltoft S. A phosphoserine-regulated docking site in the protein kinase RSK2 that recruits and activates PDK1. EMBO J. 2000 Jun 15;19(12):2924-34. PubMed PMID: 10856237; PubMed Central PMCID: PMC203368.

Frodin M, Antal TL, Dummler BA, Jensen CJ, Deak M, Gammeltoft S (2002). "A phosphoserine/threonine-binding pocket in AGC kinases and PDK1 mediates activation by hydrophobic motif phosphorylation." Embo J 21(20):5396-5407.

Filippa N, Sable CL, Hemmings BA, Van Obberghen E.Effect of phosphoinositide-dependent kinase 1 on protein kinase B translocation and its subsequent activation.Mol Cell Biol. 2000 Aug;20(15):5712-21.PubMed [citation] PMID: 10891507, PMCID: PMC86048.

Fruman DA, Meyers RE, Cantley LC.Phosphoinositide kinases.Annu Rev Biochem. 1998;67:481-507. Review.PubMed [citation] PMID: 9759495.

Garnett M, Marais, M. Guilty as charged: B-RAF is a human oncogene, Cancer Cell, Volume 6, Issue 4, October 2004, Pages 313-319, ISSN 1535-6108, 10.1016/j.ccr.2004.09.022.

Galandiuk S, Wieand HS, Moertel CG, Cha SS, Fitzgibbons RJ Jr, Pemberton JH, Wolff BG.Patterns of recurrence after curative resection of carcinoma of the colon and rectum.Surg Gynecol Obstet. 1992 Jan;174(1):27-32.PubMed [citation] PMID: 1729745.

García AL, Ruhl R, Schweigert FJ.Retinoid concentrations in the mouse during postnatal development and after maternal vitamin A supplementation.Ann Nutr Metab. 2005 Sep-Oct;49(5):333-41. Epub 2005 Aug 19.PubMed [citation] PMID: 16113519.

Harrison EH, Hussain MM.Mechanisms involved in the intestinal digestion and absorption of dietary vitamin A.J Nutr. 2001 May;131(5):1405-8. Review.PubMed [citation] PMID: 11340090.

Heslin MJ, Yan J, Johnson MR, Weiss H, Diasio RB, Urist MM.Role of matrix metalloproteinases in colorectal carcinogenesis.Ann Surg. 2001 Jun;233(6):786-92.PubMed [citation] PMID: 11371737, PMCID: PMC1421321.

Heukamp I, Kilian M, Gregor JI, Neumann A, Jacobi CA, Guski H, Schimke I, Walz MK, Wenger FA.Effects of the antioxidative vitamins A, C and E on liver metastasis and intrametastatic lipid peroxidation in BOP-induced pancreatic cancer in Syrian hamsters.Pancreatology. 2005;5(4-5):403-9. Epub 2005 Jun 28.PubMed [citation] PMID: 15985764.

Hinault C, Mothe-Satney I, Gautier N, Lawrence JC Jr, Van Obberghen E. Amino acids and leucine allow insulin activation of the PKB/mTOR pathway in normal adipocytes treated with wortmannin and in adipocytes from db/db mice. FASEB J.2004 Dec;18(15):1894-6. Epub 2004 Oct 12. PubMed PMID: 15479767.

Huang S, Van Arsdall M, Tedjarati S, McCarty M, Wu W, Langley R, Fidler IJ.Contributions of stromal metalloproteinase-9 to angiogenesis and growth of human ovarian carcinoma in mice.J Natl Cancer Inst. 2002 Aug 7;94(15):1134-42.PubMed [citation] PMID: 12165638.

Huang W, Ju T, Hung M, Chen C. Phosphorylation of CBP by IKKα Promotes Cell Growth by Switching the Binding Preference of CBP from p53 to NF-κB, Molecular Cell, Volume 26, Issue 1, 13 April 2007, Pages 75-87, ISSN 1097-2765, 10.1016/j.molcel.2007.02.019.

Huang Y, Pettaway C, Uehara H, Bucana C, Fidler I (2001). "Blockade of NF-kB activity in human prostate cancer cells is associated with suppression of angiogenesis, invasion, and metastasis." Oncogene 20: 4188-4197.

Hughes PJ, Zhao Y, Chandraratna RA, Brown G. Retinoid-mediated stimulation of steroid sulfatase activity in myeloid leukemic cell lines requires RARalpha and RXR and involves the phosphoinositide 3-kinase and ERK-MAP kinase pathways. J Cell Biochem. 2006 Feb 1;97(2):327-50. PubMed PMID: 16178010.

Ihle NT, Powis G, Kopetz S.PI-3-Kinase inhibitors in colorectal cancer.Curr Cancer Drug Targets. 2011 Feb;11(2):190-8. Review.PubMed [citation] PMID: 21158718.

Ihle NT, Powis G.Inhibitors of phosphatidylinositol-3-kinase in cancer therapy.Mol Aspects Med. 2010 Apr;31(2):135-44. Epub 2010 Feb 20. Review.PubMed [citation] PMID: 20176047, PMCID: PMC2877630.

Inoue H, Fukuyama S, Matsumoto K, Kubo M, Yoshimura A. Role of endogenous inhibitors of cytokine signaling in allergic asthma. Curr Med Chem. 2007;14(2):181-9. Review. PubMed PMID: 17266577.

Kitamura M, Ishikawa Y, Moreno-Manzano V, Xu Q, Konta T, Lucio-Cazana J, Furusu A, Nakayama K. Intervention by retinoic acid in oxidative stress-induced apoptosis. Nephrol Dial Transplant. 2002;17 Suppl 9:84-7. Review. PubMed PMID:12386300.

Khalaf H, Jass J, Olsson PE. Differential cytokine regulation by NF-kappaB and AP-1 in Jurkat T-cells. BMC Immunol. 2010 May 27;11:26. PubMed PMID: 20507572; PubMed Central PMCID: PMC2889865.

Keely PJ, Westwick JK, Whitehead IP, Der CJ, Parise LV.Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K.Nature. 1997 Dec 11;390(6660):632-6.PubMed [citation] PMID: 9403696.

Koumura H, Sugiyama Y, Kunieda K, Saji S.[Significance in gene expression of matrix metalloproteinase-9, urokinase-type plasminogen activator and tissue inhibitor of metalloproteinase for metastases of gastric and/or colo-rectal cancer].Gan To Kagaku Ryoho. 1997 Jul;24 Suppl 2:324-31. Japanese. PubMed [citation] PMID: 9263524.

Layman DK. Role of leucine in protein metabolism during exercise and recovery. Can J Appl Physiol. 2002 Dec;27(6):646-63. Review. PubMed PMID: 12501002.

Lee CH, Jeona, YT, Kimb SH, Songa YS (2007). "NF-κB as a potential molecular target for cancer therapy." BioFactors 29:19-36.

Lee JW, Soung YH, Kim SY, Lee HW, Park WS, Nam SW, Kim S SH, Lee JY, Yoo NJ, Lee SH. PIK3CA gene is frequently mutated in breast carcinomas and hepatocellular carcinomas. Oncogene. 2005 Feb 17;24(8):1477-80. PubMed PMID: 15608678.

Lee M, Han S, Jiang S, Park J, Kim S (2000) "Differential effects of retinoic acid on growth and apoptosis in human colon cancer cell lines associated with the induction of retinoic acid receptor beta". Biochem Pharm 59:485–496.

Lee S, Choi EJ, Jin C, Kim DH. Activation of PI3K/Akt pathway by PTEN reduction and PIK3CA mRNA amplification contributes to cisplatin resistance in an ovarian cancer cell line. Gynecol Oncol. 2005 Apr;97(1):26-34. PubMed PMID: 15790433.

Leede BS, Brink C, Saag P. (1993) "Retinoic acid receptor and retinoid X receptor expression in retinoic acid-resistant human tumor cell lines." Mol Carcinogenesis 1993;8:112–122.

Legrand C, Gilles C, Zahm JM, Polette M, Buisson AC, Kaplan H, Birembaut P, Tournier JM.Airway epithelial cell migration dynamics. MMP-9 role in cell-extracellular matrix remodeling.J Cell Biol. 1999 Jul 26;146(2):517-29.PubMed [citation] PMID: 10427102, PMCID: PMC3206576.

Liu X, Chan SY, Ho PC.Comparison of the in vitro and in vivo effects of retinoids either alone or in combination with cisplatin and 5-fluorouracil on tumor development and metastasis of melanoma.Cancer Chemother Pharmacol. 2008 Dec;63(1):167-74. Epub 2008 May 9.PubMed [citation] PMID: 18465132.

Liu ZL, Luo JM, Wang FX, Zhang XJ, Du XY, Yao L, Dong ZR.[Improved RT-PCR for detection of PML/RARalpha fusion gene in rapid diagnosis of acute promyelocyticleukemia]. Zhongguo Shi Yan Xue Ye Xue Za Zhi. 2003 Dec;11(6):587-90. Chinese. PubMed [citation] PMID: 14706140.

Lu Y, Hong TG, Jin M, Yang JH, Suh SJ, Piao DG, Ko HK, Seo CS, Chang YC, Kim CH, Son JK, Chang HW. Saucerneol G, a new lignan, from Saururus chinensis inhibits matrix metalloproteinase-9 induction via a nuclear factor κB and mitogen activated protein kinases in lipopolysaccharide-stimulated RAW264.7 cells. Biol Pharm Bull. 2010;33(12):1944-8. PubMed PMID: 21139230.

Martelli AM, Evangelisti C, Chiarini F, Grimaldi C, Manzoli L, McCubrey JA. Targeting the PI3K/AKT/mTOR signaling network in acute myelogenous leukemia. Expert Opin Investig Drugs. 2009 Sep;18(9):1333-49. Review. PubMed PMID: 19678801.

Marquez C, Bair SM, Smithberger E, Cherpelis BS, Glass LF.Systemic retinoids for chemoprevention of non-melanoma skin cancer in high-risk patients.J Drugs Dermatol. 2010 Jul;9(7):753-8. Review.PubMed [citation] PMID: 20677528.

McCawley LJ, Matrisian LM.Matrix metalloproteinases: they're not just for matrix anymore! Curr Opin Cell Biol. 2001 Oct;13(5):534-40. Review.PubMed [citation] PMID: 11544020.

McCubrey JA, Steelman LS, Kempf CR, Chappell WH, Abrams SL, Stivala F, Malaponte G, Nicoletti F, Libra M, Bäsecke J, Maksimovic-Ivanic D, Mijatovic S, Montalto G, Cervello M, Cocco L, Martelli AM.Therapeutic resistance resulting from mutations in Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR signaling pathways.J Cell Physiol. 2011 Nov;226(11):2762-81. doi: 10.1002/jcp.22647. Review.PubMed [citation] PMID: 21302297.

McCubrey JA, Steelman LS, Chappell WH, Abrams SL, Wong EW, Chang F, Lehmann B,Terrian DM, Milella M, Tafuri A, Stivala F, Libra M, Basecke J, Evangelisti C, Martelli AM, Franklin RA. Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. Biochim Biophys Acta. 2007 Aug;1773(8):1263-84. Epub 2006 Oct 7. Review. PubMed PMID: 17126425; PubMed Central PMCID: PMC2696318.

Mellinghoff IK, Sawyers CL (2002). "The emergence of resistance to targeted cancer therapeutics." Pharmacogenomics 3(5):603-623.

Mora A, Komander D, van Aalten DM, Alessi DR (2004). "PDK1, the master regulator of AGC kinase signal transduction." Semin Cell Dev Biol 15(2):161-170.

Na S, Kang B, Chung S, Han S, Ma X (1999). "Retinoids inhibit interleukin-12 production in macrophages through physical associations of retinoid X receptor and NFκB." J Biol Chem 274:7674–7680.

Nandan MO, Yang VW. An Update on the Biology of RAS/RAF Mutations in Colorectal Cancer. Curr Colorectal Cancer Rep. 2011 Jun 1;7(2):113-120. PubMed PMID: 21625338; PubMed Central PMCID: PMC3103074.

Ohmori Y, Hamilton TA. The interferon-stimulated response element and a kappa B site mediate synergistic induction of murine IP-10 gene transcription by IFN-gamma and TNF-alpha. J Immunol. 1995 May 15;154(10):5235-44. PubMed PMID: 7730628.

Okuno M, Kojima S, Matsushima-Nishiwaki R, Tsurumi H, Muto Y, Friedman SL, Moriwaki H.Retinoids in cancer chemoprevention.Curr Cancer Drug Targets. 2004 May;4(3):285-98. Review.PubMed [citation] PMID: 15134535.

Papi A, Bartolini G, Ammar K, Guerra F, Ferreri AM, Rocchi P, Orlandi M.Inhibitory effects of retinoic acid and IIF on growth, migration and invasiveness in the U87MG human glioblastoma cell line.Oncol Rep. 2007 Oct;18(4):1015-21.PubMed [citation] PMID: 17786368.

Park EY, Wilder ET, Chipuk JE, Lane MA.Retinol decreases phosphatidylinositol 3-kinase activity in colon cancer cells.Mol Carcinog. 2008 Apr;47(4):264-74.PubMed [citation] PMID: 17918208.

Park EY, Wilder ET, Lane MA.Retinol inhibits the invasion of retinoic acid-resistant colon cancer cells in vitro and decreases matrix metalloproteinase mRNA, protein, and activity levels.Nutr Cancer. 2007;57(1):66-77.PubMed [citation] PMID: 17516864.

Park EY, Dillard A, Williams EA, Wilder ET, Pepper MR, Lane MA.Retinol inhibits the growth of all-trans-retinoic acid-sensitive and all-trans-retinoic acid-resistant colon cancer cells through a retinoic acid receptor-independent mechanism.Cancer Res. 2005 Nov 1;65(21):9923-33.PubMed [citation] PMID: 16267017.

Raynaud FI, Eccles S, Clarke PA, Hayes A, Nutley B, Alix S, Henley A, Di-Stefano F, Ahmad Z, Guillard S, Bjerke LM, Kelland L, Valenti M, Patterson L, Gowan S, de Haven Brandon A, Hayakawa M, Kaizawa H, Koizumi T, Ohishi T, Patel S, Saghir N, et al..Pharmacologic characterization of a potent inhibitor of class I phosphatidylinositide 3-kinases.Cancer Res. 2007 Jun 15;67(12):5840-50.PubMed [citation] PMID: 17575152.

Roeb E, Dietrich CG, Winograd R, Arndt M, Breuer B, Fass J, Schumpelick V, Matern S.Activity and cellular origin of gelatinases in patients with colon and rectal carcinoma differential activity of matrix metalloproteinase-9.Cancer. 2001 Nov 15;92(10):2680-91.PubMed [citation] PMID: 11745204.

Riojas R, Kikani C, Wang C, Mao X, Zhou L, Langlais P, Hu D, Roberts J, Dong L, Liu F (2006). "Fine Tuning PDK1 Activity by Phosphorylation at Ser163*." The Journal of Biological Chemistry 281(31):21588–21593.

Roskell DE, Biddolph SC, Warren BF.Apparent deficiency of mucosal vascular collagen type IV associated with angiodysplasia of the colon.J Clin Pathol. 1998 Jan;51(1):18-20.PubMed [citation] PMID: 9577365, PMCID: PMC500424.

Sabatini DM. mTOR and cancer: insights into a complex relationship. Nat Rev Cancer. 2006 Sep;6(9):729-34. Epub 2006 Aug 17. Review. PubMed PMID: 16915295.

Samuels Y, Zhu J, Lengauer C.Inhibiting phosphoinositide 3-kinases.Cancer Biol Ther. 2005 May;4(5):546-7. Epub 2005 May 15. No abstract available. PubMed [citation] PMID: 15908801.

Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science. 2005 Feb 18;307(5712):1098-101.PubMed PMID: 15718470.

Scheele J, Stang R, Altendorf-Hofmann A, Paul M.Resection of colorectal liver metastases.World J Surg. 1995 Jan-Feb;19(1):59-71.PubMed [citation] PMID: 7740812

Schwabe, Robert. Adenovlral (AD5) delivery of wild type IkB kinase B(IKKB) but not ad5ikka restored the defective IKB/NF-KB signaling pathway in HT-29 cells Gastroenterology Volume: 118 Issue: 4 ISSN: 0016-5085.

Seo AM, Hong SW, Shin JS, Park IC, Hong NJ, Kim DJ, Lee WK, Lee WJ, Jin DH, Lee MS. Sulindac induces apoptotic cell death in susceptible human breast cancer cells through, at least in part, inhibition of IKKbeta. Apoptosis. 2009 Jul;14(7):913-22. PubMed PMID: 19526344.

Shen PF.Molecular mechanisms of chemoprevention and therapy of cancer by retinoids.Front Biosci. 2004 Sep 1;9:2663-70. Review.PubMed [citation] PMID: 15358589.

Steelman LS, Navolanic PM, Sokolosky ML, Taylor JR, Lehmann BD, Chappell WH, Abrams SL, Wong EW, Stadelman KM, Terrian DM, Leslie NR, Martelli AM, Stivala F, Libra M, Franklin RA, McCubrey JA. Suppression of PTEN function increases breast cancer chemotherapeutic drug resistance while conferring sensitivity to mTORinhibitors. Oncogene. 2008 Jul 3;27(29):4086-95. Epub 2008 Mar 10. PubMed PMID:18332865.

Stein RC.Prospects for phosphoinositide 3-kinase inhibition as a cancer treatment.Endocr Relat Cancer. 2001 Sep;8(3):237-48. Review.PubMed [citation] PMID: 11566615.

Stephens L, Williams R, Hawkins P.Phosphoinositide 3-kinases as drug targets in cancer.Curr Opin Pharmacol. 2005 Aug;5(4):357-65. Review.PubMed [citation] PMID: 15963759.

Storz P, Toker A. 3'-phosphoinositide-dependent kinase-1 (PDK-1) in PI 3-kinase signaling. Front Biosci. 2002 Apr 1;7:d886-902. Review. PubMed PMID: 11897568.

Szondy Z, Reichert U, Fésüs L. Retinoic acids regulate apoptosis of T lymphocytes through an interplay between RAR and RXR receptors. Cell Death Differ. 1998 Jan;5(1):4-10. Review. PubMed PMID: 10200440.

Tamburini J, Green AS, Bardet V, Chapuis N, Park S, Willems L, Uzunov M, Ifrah N, Dreyfus F, Lacombe C, Mayeux P, Bouscary D. Protein synthesis is resistant to rapamycin and constitutes a promising therapeutic target in acute myeloid leukemia. Blood. 2009 Aug 20;114(8):1618-27. Epub 2009 May 20. PubMed PMID: 19458359.

Tominaga T, Sakabe T, Koyama Y, Hamano K, Yasutomi M, Takahashi T, Kodaira S, Kato T, Ogawa N.Prognostic factors for patients with colon or rectal carcinoma treated with resection only. Five-year follow-up report.Cancer. 1996 Aug 1;78(3):403-8.PubMed [citation] PMID: 8697383.

Van Antwerp D. J., Martin S. J., Kafri T., Green D. R., Verma I. M. (1996) Suppression of TNF-α-induced apoptosis by NF-κB. Science 274:787–789.

Wang CC, Cirit M, Haugh JM. PI3K-dependent cross-talk interactions converge with Ras as quantifiable inputs integrated by Erk. Mol Syst Biol. 2009;5:246. Epub 2009 Feb 17. Erratum in: Mol Syst Biol. 2011;7:524. PubMed PMID: 19225459; PubMed Central PMCID: PMC2657535.

Wang, C.Y., Cusack, J.C., Jr., Liu, R., and Baldwin, A.S., Jr. 1999. Control of inducible chemoresistance: enhanced anti-tumor therapy through increased apoptosis by inhibition of NF-κB. Nat. Med. 5:412–417.

Weng CJ, Chau CF, Hsieh YS, Yang SF, Yen GC. Lucidenic acid inhibits PMA-induced invasion of human hepatoma cells through inactivating MAPK/ERK signal transduction pathway and reducing binding activities of NF-kappaB and AP-1. Carcinogenesis. 2008 Jan;29(1):147-56. Epub 2007 Nov 16. PubMed PMID: 18024477.

Weinzweig J, Tattini C, Lynch S, Zienowicz R, Weinzweig N, Spangenberger A, Edstrom L.Investigation of the growth and metastasis of malignant melanoma in a murine model: the role of supplemental vitamin A.Plast Reconstr Surg. 2003 Jul;112(1):152-8; discussion 159 61.PubMed [citation] PMID: 12832888.

Wishart M, Dixon J (2002). "PTEN and myotubularin phosphatases: from 3-phosphoinositide dephosphorylation to disease." Trends in Cell Biology 12(12):579-585.

Wu K, Zhang Y, Xu XC, Hill J, Celestino J, Kim HT, Mohsin SK, Hilsenbeck SG, Lamph WW, Bissonette R, Brown PH. The retinoid X receptor-selective retinoid, LGD1069, prevents the development of estrogen receptor-negative mammary tumors in transgenic mice. Cancer Res. 2002 Nov 15;62(22):6376-80. PubMed PMID: 12438218.

Wymann MP, Bulgarelli-Leva G, Zvelebil MJ, Pirola L, Vanhaesebroeck B, Waterfield MD, Panayotou G.Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction.Mol Cell Biol. 1996 Apr;16(4):1722-33.PubMed [citation] PMID: 8657148, PMCID: PMC231159.

Yan M, Xu Q, Zhang P, Zhou XJ, Zhang ZY, Chen WT. (2010) "Correlation of NF-kappaB signal pathway with tumor metastasis of human head and neck squamous cell carcinoma." BMC Cancer 10:437.

Yang XD, Tajkhorshid E, Chen LF. Functional interplay between acetylation and methylation of the RelA subunit of NF-kappaB. Mol Cell Biol. 2010 May;30(9):2170-80. Epub 2010 Feb 16. PubMed PMID: 20160011; PubMed Central PMCID: PMC2863596.

Yin Y, Shen WH (2008). "PTEN: a new guardian of the genome." Oncogene 27(41): 5443-5453.

Yuan TL, Cantley LC (2008). "PI3K pathway alterations in cancer: variations on a theme." Oncogene 27(41):5497-5510.

Zanotto-Filho A, Cammarota M, Gelaina DP, Oliveiraa RB, Delgado-Cañedoc A, Dalmolina RJ, Pasqualia MA, Moreira JC. (2008). "Retinoic acid induces apoptosis by a non-classical mechanism of ERK1/2 activation." Toxicol In Vitro 22(5):1205-1212.

Zanotto-Filho A, Gelain DP, Schröder R, Souza LF, Pasquali MA, Klamt F, Moreira J (2009). "The NF κ B-mediated control of RS and JNK signaling in vitamin A-treated cells: duration of JNK-AP-1 pathway activation may determine cell death or proliferation." Biochem Pharmacol. 77(7):1291-1301.

Zeng L, An S, Goetzl EJ.Regulation of expression of matrix metalloproteinase-9 in early human T cells of the HSB.2 cultured line by the EP3 subtype of prostaglandin E2 receptor.J Biol Chem. 1996 Nov 1;271(44):27744-50.PubMed [citation] PMID: 8910368.

Zeng ZS, Cohen AM, Guillem JG.Loss of basement membrane type IV collagen is associated with increased expression of metalloproteinases 2 and 9 (MMP-2 and MMP-9) during human colorectal tumorigenesis.Carcinogenesis. 1999 May;20(5):749-55.PubMed [citation] PMID: 10334190.

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