ROLE OF RETINOL IN COLON CANCER INVASION AND METASTASIS

THESIS

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DEDICATION

To my wonderful family- Mom, Dad, Sarah, Missy, and Maggie May.

Thank you for all of your love and support.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

TABLE OF FIGURES

Figure **Page**

I. INTRODUCTION AND LITERATURE REVIEW

COLORECTAL CANCER

Colorectal cancer is the third most common cancer and second most common cause of death due to cancer in both men and women in the United States (A.C.S. 2009). Several inherent risk factors of colon cancer exist, including age 50 years and older, genetic mutations, those with a personal or family history of inflammatory bowel disease, or those with a personal or family history of colorectal cancer and polyps [For a review see: (Waldmann et al. 2009)]. In addition, there are modifiable risk factors for colon cancer. For example, being overweight or obese is associated with a higher risk of colorectal cancer (Kim et al. 2007) and increased activation of pro-carcinogenic pathways (e.g. PI3K/Akt) in men and women (Huang et al. 2009). Similarly, physical inactivity is associated with an increased risk of colon cancer (Wolin et al. 2009). High levels of physical activity may decrease the risk of colon cancer among men and women by as much as 40% (Lee 2003). In addition, tobacco use (Luchtenborg et al. 2007), excessive alcohol consumption (Rehm et al. 2010), high red meat intake (Norat et al. 2005), and deficient vegetable and fruit intake (Satia et al. 2009) can also increase the risk of colorectal cancer.

The majority of colorectal cancers and deaths may be excluded by early detection and prevention via increased frequency of screening tests. Screening can prevent many cases of colon cancer by identifying precancerous adenomatous polyps, or growths, in the colon and rectum which can be removed before the development of malignant colon cancer (A.C.S. 2009). The most common treatment for colorectal cancer is surgery, usually in combination with adjuvant chemotherapy and radiation (A.C.S. 2009). Early stage detection can allow surgical removal of colon cancer and extended remission.

Although established screening techniques have progressed, rates of screening for colorectal cancer are low due to cultural and economic factors among others, which results in a significant number of colon cancer cases not detected until later stages of the disease and an increased rate of colorectal cancer related deaths. The American Cancer Society estimated 49,920 deaths due to colon cancer in 2009. Colorectal cancer mortality is generally not due to the primary tumor, but rather the metastases of the cancer, primarily to the liver (Scheele et al. 1995). The five-year survival rate of people with colorectal cancer diminishes from 68% to 11% once distant metastases form (A.C.S. 2009) which underscores the importance of cancer therapies that prevent the progression of colon cancer metastasis.

Metastasis is the pathway by which primary tumor cells travel to secondary sites and proliferate, spreading the cancer to other organs. Metastasis is composed of numerous processes including: (1) invasion or digestion of the basement membrane by cells from the primary tumor and migration of these cells through the basement membrane into the circulation (intravasation), (2) survival in circulation, (3) migration of the cells out of circulation (extravasation), (4) invasion into the secondary target site via

digestion of the extracellular matrix (ECM), and (5) metastatic cell proliferation and growth in the secondary tissue (Woodhouse et al. 1997) (See Figure 1.1). Basement membrane and ECM degradation requires proteinases that possess proteolytic activity. Proteinases are involved in tumor cell invasion at several stages of metastasis such as angiogenesis, local invasion, intravasation, and extravasation (Garbett et al. 1999). Although ECM turnover is normally regulated and homeostatically controlled, excessive proteolysis occurs during cancer invasion (Yu et al. 1997) leading to increased metastasis and reduced disease free survival. Therefore, an increased amount of attention has been placed on investigating therapies that prevent or reduce the metastatic progression of colorectal cancer. The FDA has approved of three new targeted monoclonal antibody therapies, Bevacizumab (Avastin), Cetuximab (Erbitux), and Panitumumab (Vectibix), which exert anti-angiogenic and anti-proliferative properties to slow the metastatic process (A.C.S. 2009). However, this therapy increases the risk for arterial thromboemblic events, upper respiratory infections, and complications in wound healing. In addition, adjuvant chemotherapy has been applied to treat persons with metastatic colorectal cancer. For example, oxaliplatin in combination with the Folfox7 regimen, followed by bevacizumab is administered and combined with radiation therapy (Oukkal et al. 2010). Similarly, these drugs have numerous unwanted side effects such as nausea, diarrhea, poor appetite, decreased blood cell production, pain and blistering of the palms and feet, decreased sensation, and decreased proprioception (A.C.S. 2009). Therefore alternative preventative and therapeutic treatments that have a significant effect on the progression of colon cancer with minimal side effects are in high demand.

Numerous studies have investigated the ability of bioactive dietary components including fiber (Dahm et al. 2010), folate (Stelmaszuk et al. 2009), calcium (Weingarten et al. 2008), vitamin D (Cross et al. 2009), and omega-3 fatty acids (Wendel et al. 2009) to prevent and treat colon cancer. These dietary components may possess chemopreventive or chemotherapeutic properties and reduced detrimental side effects. The metabolite of vitamin A, *all-trans* retinoic acid (ATRA), has been extensively studied regarding its ability to prevent or treat various types of cancer (Frankenberger et al. 2001; Arrieta et al. 2010; Charoensit et al. 2010; Dutta et al. 2010; Tallman 2010). In contrast, the dietary form of vitamin A has received little attention yet holds promise as a chemopreventative and chemotherapeutic agent. Hence, we evaluated the ability of dietary vitamin A to prevent metastasis and slow the progression of human colorectal cancer.

RETINOIDS

Vitamin A Metabolism

The retinoids, a group of compounds consisting of vitamin A (retinol), its natural metabolites (ATRA), and several synthetic compounds, have been shown to act as cancer chemopreventive agents (Bukhari et al. 2007; Lu et al. 2008; So et al. 2008). The diet contains vitamin A in two forms: (1) previtamin A carotenoids in plant-derived food sources and (2) preformed vitamin A as retinol and retinyl esters in animal-derived food sources. Retinyl esters are cleaved within the intestinal lumen to yield retinol. Upon

absorption from the gut, retinol is esterified, forming retinyl esters that are incorporated into chylomicrons, bound to retinol-binding proteins (RBPs), and sent to the liver, the main retinol storage site (Vogel 1999). In addition to the retinol present in the intestinal lumen, retinol can reach colonocytes via circulation bound to RBPs or as free retinol (Harrison et al. 2001).

In most cells, retinol is either esterified for storage or metabolized to ATRA. ATRA is the most widely studied and bioactive metabolite of retinol (Vogel 1999) which acts as a transcription factor regulating cell growth and induces differentiation. Specifically, ATRA binds to retinoic acid receptors (RAR), which heterodimerize with retinoid X receptors (RXR) and bind to retinoic acid response elements (RARE) in the promoter regions of ATRA-responsive genes, inducing gene expression. Serum levels of ATRA are considerably lower than serum retinol and have been reported to range from 1-14 nM (Blaner et al. 1985).

Serum retinol levels are between 0.5-2 μM (Smith et al. 1971) and do not increase following dietary vitamin A supplementation. As mentioned above, the intestinal lumen, including the colonocytes, is primarily exposed to retinol via the diet. To our knowledge, intraluminal concentrations of retinol in the colon have not been measured but they can be raised by increasing dietary vitamin A levels. Very little data exists concerning normal hepatic retinol levels. However, elevated intestinal lumen and hepatic concentrations of retinol have been attained by dietary vitamin A supplementation [for review see: (Loerch et al. 1979; Russell 2000)]. Specifically, mice consuming milk from dams fed a diet supplemented with 589,091 IU vitamin A/kg until 21 days of age and then the supplemented diet itself until 65 days of age reported retinol concentrations as

high as 90.8 μM (26.0 ± 3.2 μg/g) (Garcia et al. 2005). In addition, consumption of 6,000 IU/kg diet by rats resulted in a hepatic retinol concentration of 6.4 μM (Schmidt et al. 2003).

Retinoids and Metastasis

Retinoids have been shown to inhibit metastasis in a variety of model systems. Specifically, dietary retinyl palmitate decreased malignant melanoma metastasis in mice (Weinzweig et al. 2003). Similarly, retinol decreased hepatic metastases in a hamster model of pancreatic ductal carcinoma (Heukamp et al. 2005). In addition, treatment with a combination of cisplatin (chemotherapy drug) and 13-*cis*-retinoic acid 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).

Retinoids may reduce invasion and metastasis by decreasing the protein levels or activity of the proteolytic enzymes called matrix metalloproteinases (MMPs) that play a key role in tissue remodeling and metastasis. For example, ATRA reduces breast cancer cell invasion by decreasing matrix metalloproteinase-9 (MMP-9) activity (Liu et al. 2003). Also, ATRA has been shown to decrease MMP-9 and increase tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) gene expression in murine lung alveolar carcinoma (Andela et al. 2004). Application of the synthetic retinoid N-(-4-hydroxyphenyl) retinamide (4-HPR) in combination with cholecalciferol (vitamin D_3) resulted in growth inhibition and decreased MMP activity in RWPE-1 human prostate epithelial cells (Tokar et al. 2006). In addition, bexarotene, a novel oral synthetic retinoid, decreased the migration and invasion of A549 adenocarcinomic human alveolar

basal eptithelial cells by reducing MMP levels and increasing TIMP secretion (Yen et al. 2006). 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). In addition, HF alone, or in combination with the peroxisome proliferator-activated receptor-γ (PPARγ) ligand ciglitazone, inhibited cell growth as well as tissue invasion ability via decreased MMP-2 and MMP-9 expression and activity in glioblastoma U87MG and melanoma G361 cells (Papi et al. 2009). Application of ATRA reduced the invasive potency of C643 and HTH74 metastatic thyroid cancer cells via down-regulation of the invasion-related proteins urokinase type plasminogen activator (uPA), uPA receptor, and matrix metalloproteinase-2 (MMP-2) (Lan et al. 2009).

As can be seen from the literature cited above, ATRA is the most widely studied and bioactive metabolite of retinol (Vogel 1999) and is believed to mediate the effects of retinoids on carcinomas. However, as cancer progresses, tumors frequently become resistant the inhibitory effects of ATRA. ATRA resistance is caused by a defect in RAR induction in response to ATRA (Sonneveld et al. 1998; Nicke et al. 1999; Lee et al. 2000) due to methylation of the RARE in the RAR's promoter region. ATRA resistance diminishes the effectiveness of ATRA as cancer chemotherapy. Therefore, it is more relevant to examine the effects of retinol on cell invasion and metastasis because colon cells are primarily exposed to retinol and retinol is stored in the liver, the primary site of colorectal cancer metastasis.

Our laboratory has shown that retinol possesses a novel anti-metastatic function. Using two ATRA-resistant colon cancer cell lines, our laboratory has found that both cell

migration and invasion were inhibited by retinol in a dose-responsive manner (Park et al. 2007). 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). Thus, the effects of retinol are ATRA and RAR independent. In addition, research in our laboratory has shown that retinol inhibits the activity of phosphatidylinositol 3-kinase, a key regulatory enzyme in the metastatic process (Park et al. 2008).

PROTEINS INVOLVED IN METASTASIS

Phosphatidylinositol 3-kinase (PI3K)

Phosphatidylinositol 3-kinases (PI3K) have a key role in the regulation of many cellular processes including proliferation, cell survival, carbohydrate metabolism, and motility (Stein 2001) (Figure 1.2A). PI3K is somatically mutated in over 25% of colorectal tumors and amplification of genomic regions containing PI3K genes has been reported (Samuels et al. 2005). Activation of PI3K is associated with increased cell invasion and tumor metastasis (Keely et al. 1997; Shaw et al. 1997; Stephens et al. 2005). In addition, elevated PI3K activity has been associated with an invasive phenotype in human colon cancer (Stephens et al. 2005). Class 1A PI3Ks, the class expressed in human colon cancers including the tumor from which the HCT-116 cell line was derived (Shao et al. 2004), are heterodimers composed of p110α catalytic and p85 regulatory subunits and can be activated by recruitment to the cell surface by growth factor receptor tyrosine kinases (Vanhaesebroeck et al. 1999; Cantley 2002).

Active PI3Ks catalyze the phosphorylation of the 3-OH position of the inositol head group of phosphatidylinositol 4, 5-bisphosphate (PIP_2) converting this compound to phosphatidylinositol 3,4,5-triphosphate (PIP_3) (Samuels et al. 2005). Our lab recently reported that retinol treatment reduces the activity of PI3K (Park et al. 2008). This effect was dose-responsive, independent of RAR and ATRA-mediated mechanisms. In addition, our lab has also shown that expression of constitutively active (ca) PI3K in human colon cancer cells blocks the ability of retinol to decrease cell invasion indicating the effect of retinol on colon cancer cell invasion is mediated by PI3K (Figure 1.3).

Comparison of the electrostatic potential surfaces of retinol, phosphatidylinositol (PI), and the PI3K inhibitor, wortmannin, revealed retinol, with its tail bent into a ring, had a more similar electrostatic potential surface profile to wortmannin than to phosphatidylinositol (PI) (Park et al. 2008). Previous work has shown that wortmannin interacts with ATP binding site of the p110α catalytic subunit of PI3K to inhibit activity (Wymann et al. 1996). Therefore, it is likely that retinol interacts with PI3K in a manner similar to that of wortmannin. 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. This modeling shows that retinol interacts with p110α at the ATP binding site of PI3K, similar to wortmannin, but unlike the previous gas-phase modeling, retinol interacts with a linear tail conformation. This supports the theory that retinol behaves similarly to wortmannin to inhibit PI3K activity. More data is needed to determine the precise conformation of retinol when it interacts with PI3K.

Protein Kinase B (Akt)

Akt is a serine-protein kinase, downstream of PI3K signaling, that plays a critical role in cellular survival, cell cycle, motility, growth, and metastasis (Rhodes et al. 2008) (Figure 1.2A). PI3K activates PIP_3 which can then act a docking site for Akt and PIP_3K-dependent protein kinase-1 (PDK1) interactions. PDK1 can then activate Akt via phosphorylation at the Ser473 and Thr308 residues; phosphorylation of the Thr308 residue is essential for activity. Once stimulated, Akt is able to activate or inhibit numerous downstream targets such as glycogen synthase kinase-3β (GSK3β) which in turn regulate numerous cellular processes. An activating mutation in the pleckstrin homology domain of Akt has been identified in human breast, ovarian, and colorectal cancers, suggesting a direct role of Akt in human cancers (Carpten et al. 2007). In addition, amplification and mutation of epidermal growth factor receptor (EGFR), PI3K, receptor tyrosine kinases, loss or mutation of tumor suppressor protein (PTEN), or mutation of Akt itself can result in increased Akt signaling in tumor cells (Rhodes et al. 2008). Increased Akt activation has been reported to correlate with cell proliferation, apoptosis inhibition, invasion, and metastasis (Itoh et al. 2002). Previous studies indicate that Akt over-expression/activation is highly correlated to human colorectal cancer (Johnson et al. 2010) and suggest that apoptosis inhibition during sporadic colon cancer carcinogenic process can be partially attributed to Akt (Roy et al. 2002). Moreover, Akt has been shown to regulate the invasion of cancer cells. For example, Akt regulated the invasive properties of highly metastatic HT1080 fibrosarcoma cells by modulating the expression of MMP-9 (Kim et al. 2001). In addition, it has been reported that hyperactivation and increased levels of Akt coincide with progression to metastasis and

increased metastatic capability in 21T breast cancer cells (Qiao et al. 2007). Similarly, Akt phosphorylation is inversely related to lymphatic invasion or lymph node metastasis in human gastric carcinomas (Nam et al. 2003).

Our research shows that treatment with retinol decreases Akt-phosphorylation (Figure 1.4A). In addition, transfection of HCT-116 cells with a constitutively active (ca) Akt construct blocked the ability of retinol to decrease Akt phosphorylation and inhibit cell invasion (Figure 1.4B and C). In summary, our data thus far show that retinol inhibits cell invasion independent of induction of gene transcription and translation through a novel mechanism involving PI3K/Akt inactivation.

Glycogen Synthase Kinase-3β (GSK3β)

When Akt is activated, Akt is able to phosphorylate downstream targets, such as the multifunctional serine/threonine kinase GSK3β (Figure 1.2A). More than 40 proteins are substrates of GSK3β including transcription factors, cell cycle/survival regulators and oncogenic/pro-oncogenic proteins (Doble et al. 2003; Jope et al. 2004). Activation of GSK3β depends on the phosphorylation of the Tyr216 residue (Wang et al. 1994). In contrast, phosphorylation of the Ser9 residue of GSK3β by Akt inactivates this enzyme (Stambolic et al. 1994). Inhibition of GSK3β activity forces an increase in levels of cytosolic and, consequently, nuclear β-catenin. Nuclear β-catenin then binds to T cell factor/ lymphoid enhancer factor (TCF/LEF) transcription factors, forming a potent transcription regulatory complex, which alters transcription of target genes.

Reported targets of β-catenin in the colon include genes involved in cell proliferation such as c-myc and cyclin D_1, and metastasis such as matrix

metalloproteinase-7 (MMP-7) (Batlle et al. 2002; van de Wetering et al. 2002) (Figure 1.2A). In the absence of Akt inhibition, GSK3β is ubiquitously expressed and constitutively active (Woodgett 1994; Cook et al. 1996; Harwood 2001). GSK3β cooperates with various proteins and components to promote proteasomal degradation of β-catenin (Hart et al. 1998; Ikeda et al. 1998; Sakanaka et al. 1998) and thus regulation of target genes affected by the β-catenin-TCF/LEF complex. Mutations in Akt, GSK3β, or β-catenin that result in inhibition of degradation of β-catenin can cause high levels of stabilized β-catenin accumulation. These mutations translate to high levels of β-catenin-TCF/LEF complexes in the nucleus, uncontrolled activation, and deregulation of the expression of target genes which promotes carcinogenesis via increased proliferation, migration, and tumor vascularization (Dihlmann et al. 2005). This cascade of events has been implicated as a key component of colon, hepatocellular carcinomas, and prostate cancer (Barker et al. 2000; Bienz et al. 2000; Polakis 2000).

Our laboratory has shown that treatment with retinol at various time points does not significantly increase GSK3β activity, specifically by decreasing Ser9 GSK3β phosphorylation. Therefore, since retinol may not directly affect GSK3β activity levels, the research described herein will not use this protein to further examine the mechanism by which retinol decreases invasion.

Alternatively, when Akt is activated it has been shown to phosphorylate the IKK complex (Ozes et al. 1999; Romashkova et al. 1999; Agarwal et al. 2005) which results in the downstream activation of the transcription modulator, nuclear factor-kappa beta (NF-κB) and subsequent NF-κB mediated expression of MMP-9 (Bond et al. 1998; Bauerle et al. 2010) (Figure 1.2A). NF-κB is not a focus of the current study; however due to the

reported regulation of MMP-9 and consequential invasion, it presents a possible alternative pathway of future interest.

β-catenin

β-catenin is a protein whose function in cell signaling varies, depending on its location. β-catenin in the membrane-bound adherens complex functions in cell to cell adhesion whereas nuclear β-catenin functions as a potent stimulator of gene transcription (Peifer et al. 2000). β-catenin exerts its nuclear function by interacting with the TCF/LEF regulatory complex, forming a transcription factor (Figure 1.2A).

As mentioned previously, reported targets of β-catenin in the colon include genes involved in cell proliferation such as c-myc and cyclin D_1, and metastasis such as MMP-7 (Batlle et al. 2002; van de Wetering et al. 2002). Retinoids have been shown to decrease the levels and activity of the β-catenin mediated signaling pathways (Easwaran et al. 1999; Ara et al. 2004; Eisinger et al. 2007; Sakabe et al. 2007). Our laboratory has found similar results, which showed retinol treatment decreased β-catenin protein levels *in vitro* (Dillard et al. 2007).

Cytosolic β-catenin can be translocated to the nucleus, or targeted for proteasomal degradation. Numerous pathways direct β-catenin to the proteasome for degradation, however, mutations in the β-catenin degradation pathways are present in 70-80% of colorectal tumors (Pennisi 1998; Wong et al. 2002). Specifically, mutations in β-catenin in HCT-116 human colon cancer cells eliminate function of all β-catenin degradation pathways except a retinoid "X" receptor (RXR)-mediated pathway (Dillard et al. 2007). The HCT-116 cells express high levels of β-catenin and lack RARs, but express RXRs

(van der Leede et al. 1993). Previously, our laboratory has reported that retinol, which is not a RXR ligand, reduced nuclear β-catenin protein levels in HCT-116 cells by increasing cystolic β-catenin and proteasomal degradation via a RXR-mediated pathway (Dillard et al. 2007). In addition, the retinol-induced activation of the RXR pathway occurred independent of a RXR agonist. Application of RXR antagonist or RXRα siRNA inhibited the ability of retinol to decrease total cellular β-catenin (Dillard et al. 2007) which suggested that RXRα facilitates the ability of retinol to decrease β-catenin protein levels.

The activation function-1 (AF-1) and DNA binding domains (DBD) of RXRα bind to β-catenin (Xiao et al. 2003; Lin et al. 2004). Recently our laboratory has shown that in cells transfected with a RXRα construct lacking the AF-1 and DBD domains eliminated the ability of retinol to decrease β-catenin protein, indicating that RXRα and β-catenin binding is required for transport to the cytosol and subsequent proteasomal degradation of β-catenin (Dillard et al. 2008). Moreover, we have reported that retinol treatment induces β-catenin-RXRα binding leading to increased proteasomal degradation of β-catenin and RXRα in ATRA-resistant human colon cancer cells (Dillard et al. 2008).

Matrix Metalloproteinase-9 (MMP-9)

The metastatic process requires several steps including the invasion or digestion of the basement membrane by cells from the primary tumor and invasion into the secondary target site via digestion of the extracellular matrix (ECM) (Woodhouse et al. 1997). The digestion of the protein components of the basement membrane and ECM is performed by MMPs (Figure 1.2A). MMPs are a family of proteolytic enzymes

associated with tissue remodeling processes that occur during tumor invasion and metastasis (Harris 1990; Page 1991; Kleiner et al. 1999). MMPs are first expressed as inactive zymogens and must be cleaved to become active enzymes. MMP activity is regulated both at cleavage and total protein expression levels.

The main MMP focused on in this study, MMP-9, is a zinc-dependent extracellular gelatinase which promotes metastatic progression in colorectal cancer via highly specific degradation of ECM proteins (Bernhard et al. 1994; Legrand et al. 1999; Bergers et al. 2000; McCawley et al. 2001; Huang et al. 2002). Tumor cell invasion has been linked to MMP-9 activity (Kubota et al. 1991; Stetler-Stevenson et al. 1996). MMP-9 is over-expressed in colorectal carcinomas. It is believed that this over-expression may be part of the mechanism by which carcinoma cells metastasize (Heslin et al. 2001). Increased levels of MMP-9 expression 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; Lubbe et al. 2006). Reports have shown patients with colon carcinoma have a significant increase in levels of MMP-9 protein and up-regulation of MMP-9 transcription in tumor areas compared with noninvolved regions (Roeb et al. 2001; Herszenyi et al. 2008). The increase in MMP-9 levels and cell motility may be mediated by PI3k/Akt activity. Numerous studies have reported MMP-9 as a downstream target of PI3K/Akt activation and key protein responsible for invasion in various carcinomas (Cheng et al. 2006; Arcaro et al. 2007). Specifically, Kim et al. (2001) reported that Akt potently promoted the invasion of highly metastatic HT1080 human fibrosarcoma cells by increasing MMP-

9 production in a manner highly dependent on PI3K activity. Also, Chung et al. (2004) found that hepatitis B virus X protein-induced expression of MMP-9 in hepatocellular carcinoma cells was reduced by inhibitors of the PI3K/Akt pathway, indicating the involvement of PI3K/Akt signaling in transcriptional regulation of MMP-9. Recently, Chen et al. (2009) supported this data by reporting that activated PI3K/Akt signaling pathway was associated with frequent intrahepatic metastasis and vascular invasion through up-regulation of MMP-9 expression in human hepatocellular carcinoma.

Over-expression of MMP-9 by colon cancer cells, which is essential to the metastatic process, provides an opportunity to create therapies specifically targeting MMP-9 protein and activity. Numerous studies have reported potent inhibitors of tumor metastases via reduction of MMP-9 activity and expression involving PI3K/Akt mediated pathways (Yoon et al. 2006; Shih et al. 2007; Park et al. 2009). Our laboratory has shown that retinol treatment reduced invasion of HCT-116 colon cancer cells by decreasing MMP-9 mRNA levels, MMP-9 protein levels and gelatinase activity (Park et al. 2007). We have also shown that inhibition of MMP-9 activity via neutralizing antibodies decreased cell invasion to 19 ± 12% in HCT-116 colon cancer cells (Park et al. 2007), indicating that MMP-9 is involved in the process of cell invasion in HCT-116 colon cancer cells and retinol is able to decrease cell invasion and metastasis, at least in part, by decreasing MMP-9 activity.

Tissue Inhibitors of Matrix Metalloproteinases-1 (TIMP-1)

Tissue inhibitors of matrix metalloproteinases (TIMPs) are endogenous protease inhibitors that regulate MMP activity (Figure 1.2A). TIMPs inhibit activity by forming

high-affinity, noncovalent, and essentially irreversible complexes with MMPs (Roeb et al. 2001). Tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) is a member of the TIMP family that binds preferentially to MMP-9. Specifically, TIMP-1 inhibits MMP-9 activity by binding to pro-MMP-9 which prevents the conversion of pro-MMP-9 to active MMP-9 (Mook et al. 2004). TIMPs have been shown to inhibit cell invasion *in vitro*, tumorigenesis, metastasis *in vivo*, angiogenesis, and reduce tumor cell growth (Gomez et al. 1997). However, the mechanism by which invading tumor cells evade these highly effective MMP inhibitors is not well comprehended. Previously, retinoids have been shown to reduce cancer cell invasion via up-regulation TIMP-1 levels (Liu et al. 2003; Guruvayoorappan et al. 2008). Our laboratory found that treatment with retinol increases extracellular TIMP-1 protein levels (Park et al. 2007) which may contribute to the overall decrease in invasion observed with retinol application.

SUMMARY

In conclusion, our preliminary data show that retinol decreases the metastatic potential of ATRA resistant colon cancer cells. We hypothesize that treatment with retinol results in inhibition of the PI3K/Akt pathway which leads to decreased β-catenin levels via proteasomal degradation, and decreased transcription of genes regulated by the β-catenin-TCF/LEF complex such as cyclin D_1, c-myc, and MMPs. Ultimately, we hypothesize that a decrease in MMP-9 levels and increase in TIMP-1 protein levels result in decreased cell invasion and metastasis. A summary of our proposed mechanism of retinol action is shown in Figure 1.2B.

This thesis focuses on the study of the mechanism by which retinol acts as an inhibitor of ATRA resistant human colon cancer metastasis *in vitro* and *in vivo*. The aim of this chapter was to provide background information concerning colon cancer metastasis, chemopreventive actions of retinol, and the role of invasion-related proteins affected by retinol in colon cancer metastasis. Chapter 2 will focus on the mechanism by which retinol reduces metastasis of ATRA resistant human colon cancer cells, specifically it will examine the ability of PI3K/Akt activation to mediate the inhibitory effects of retinol on colon cancer cell invasion. Chapter 3 will build on these findings and examine the role of PI3K in the ability of dietary vitamin A supplementation to inhibit liver metastasis of colon tumors in a nude mouse xenograft model. Taken together, these studies suggest that retinol can reduce the invasion and metastasis of ATRA-resistant human colon cancer cells by inhibiting PI3K and Akt activity, thus decreasing MMP-9 activity and consequential metastasis. Chapter 4 will summarize the findings of these studies and propose future directions to further elucidate the exact mechanisms by which retinol reduces metastasis of ATRA resistant human colon cancer cells.

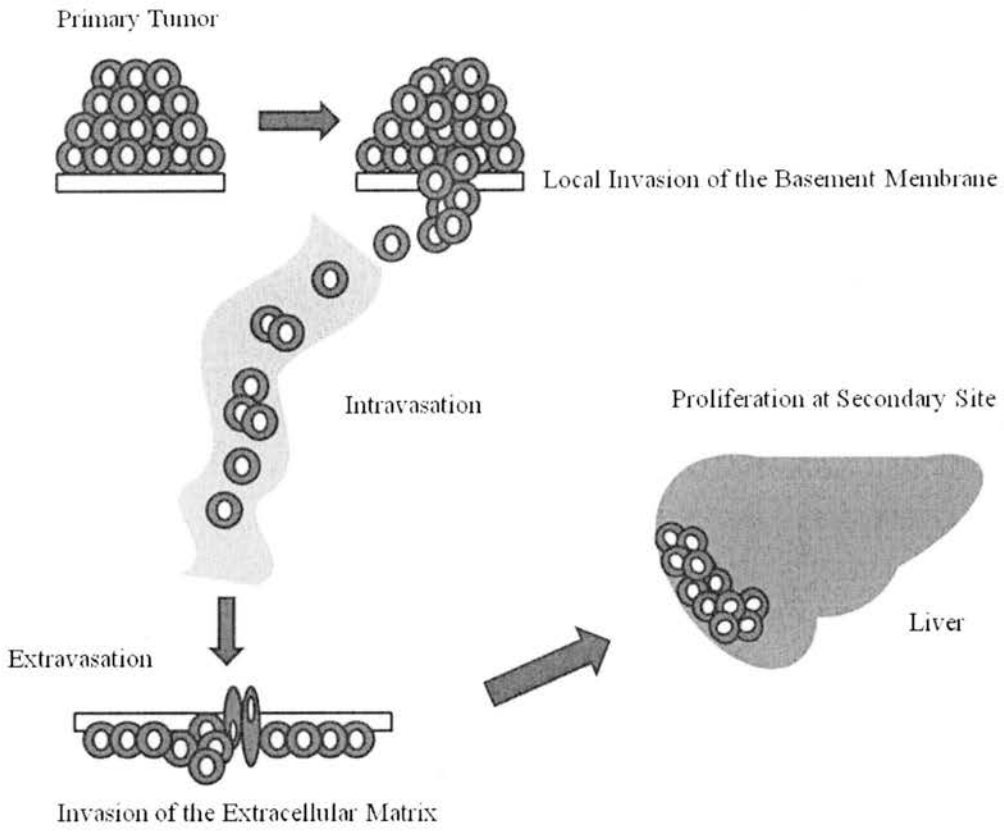

Figure 1.1. The Metastasis Process. Metastasis is composed of numerous steps including: (1) proliferation of primary tumor and local invasion of the basement membrane by detached cells, (2) intravasation in a capillary, (3) tumor cell survival in blood circulation, (4) arrest and extravasation into a secondary target site via digestion of the extracellular matrix, and (5) metastatic cell proliferation in the new environment and formation of a secondary neoplasm.

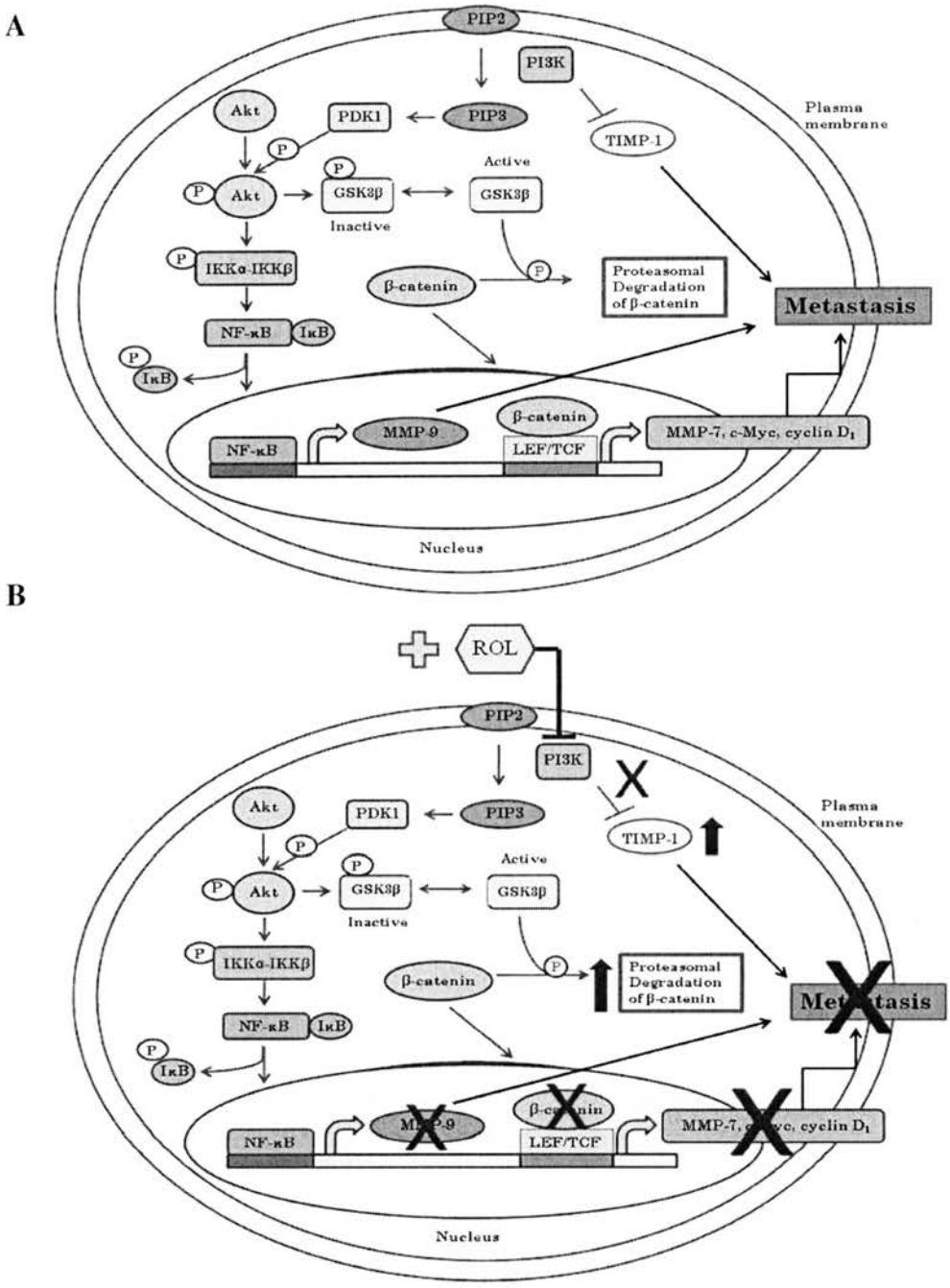

Figure 1.2. PI3K-Activated Pathways involved in colon cancer metastasis and effects of retinol on metastasis. (**A**) PI3K-activated pathway of colon cancer metastasis. (**B**) Display of the alterations in the PI3K pathway that occur when cells are treated with retinol. Decreases in inhibition, or the level of a particular mRNA or protein are indicated by an X. Increases in levels of protein or activity are indicated by an arrow. Retinol inhibits PI3K activity which results in increased TIMP-1 protein levels, increased proteasomal degradation of β-catenin, decreased expression of β-catenin and β-catenin regulated proteins, and decreased MMP-9 activity which results in an overall reduction of invasion and metastasis.

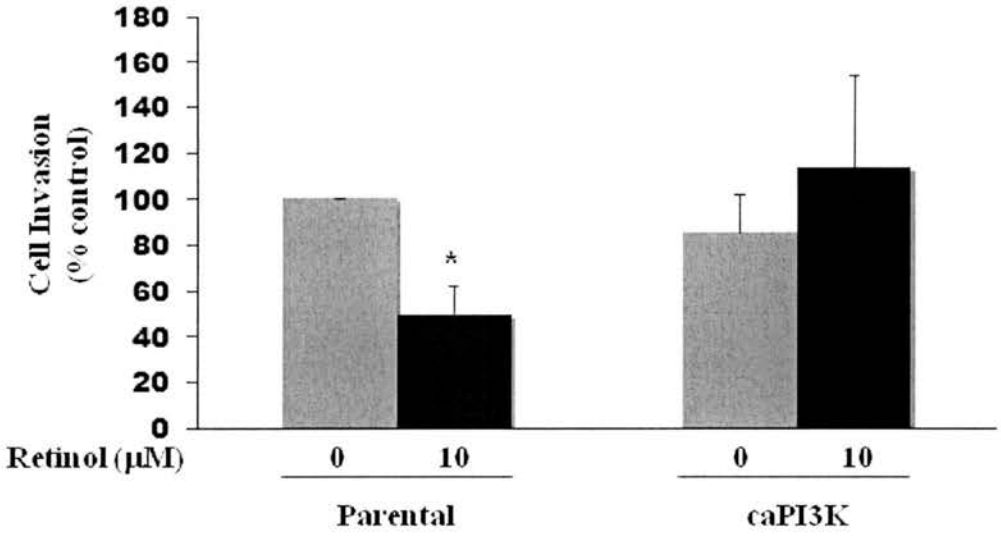

Figure 1.3. Expression of constitutively active PI3K blocks the ability of retinol to decrease colon cancer cell invasion. Parental or caPI3K-expressing HCT-116 cells were serum starved for 48 h before seeding at a density of 1 x 10^5 cells per well on Matrigel-coated Boyden chambers. The upper portion of the chambers contained 0 (ethanol vehicle control) or 10 μM retinol. The lower chamber contained 10% FBS which served as a chemoattractant. Cell invasion was measured after 24 h by staining with Wrights Stain as described in Materials and Methods. All data are reported as mean ± SEM; n=3. *Significantly different from control ($P < 0.05$).

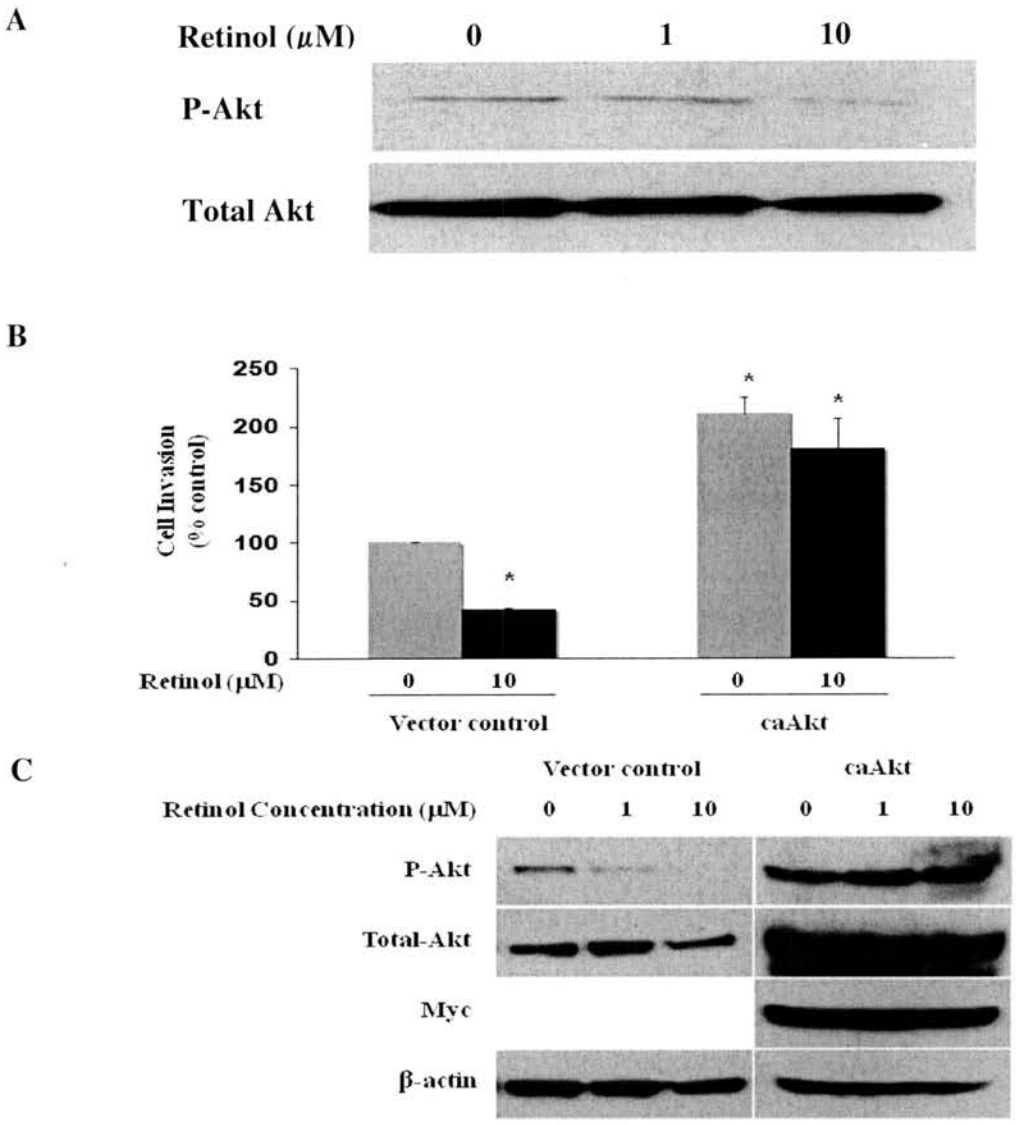

Figure 1.4. Retinol decreases Akt-phosphorylation and expression of constitutively active Akt blocks the ability of retinol to decrease colon cancer cell invasion. (**A**) Total protein was isolated from HCT-116 cells treated with 0, 1, 10 μM retinol. Samples were harvested after 50 min. Western blots were probed with phosphorylated Akt and total Akt antibodies. Data are representative of n=2. (**B**) HCT-116 cells stably transfected with caAkt plasmids (myr-Akt1) or vector control (pUSEamp+) constructs were serum starved for 48 h before seeding at a density of 1 x 10^5 cells per well on Matrigel-coated Boyden chambers. The upper portion of the chambers contained 0 (ethanol vehicle control) or 10 μM retinol. The lower chamber contained 10% FBS which served as a chemoattractant. Cell invasion was measured after 24 h by staining with Wrights Stain as described in Materials and Methods. All data are reported as mean ± SEM; n=3. *Significantly different from vector control ($P < 0.05$). (**C**) The presence of the caAkt transgene was confirmed by western blot analysis for c-myc (Upstate Biotechnology #06-549), PAkt (Cell Signaling #9275), and total Akt (Cell Signaling #9272). One representative western blot is shown.

II. PHOSPHATIDYLINOSITOL 3-KINASE ACTIVATION MEDIATES THE ABILITY OF RETINOL TO INHIBIT COLON CANCER CELL INVASION AND INVASION-ASSOCIATED PROTEINS *IN VITRO*

ABSTRACT

Dietary vitamin A supplementation decreases colorectal cancer metastasis and retinol inhibits phosphatidylinositol 3-kinase (PI3K) activity, protein kinase B (Akt) phosphorylation, and decreases β-catenin protein levels. Our objective was to determine if PI3K/Akt activation and/or β-catenin mediates the effects of retinol on colon cancer invasion and invasion-associated proteins. HCT-116 cells expressing parental or constitutively active (ca) PI3K, HCT-116 cells stably transfected with caAkt plasmids (myr-Akt1) or vector control (pUSEamp+) constructs, or HCT-116 cells transfected with a plasmid expressing mutated β-catenin resistant to proteasomal degradation (S37A mutation) or the empty plasmid vector (pcDNA3.1) were treated with 0 (vehicle control) or 10 μM retinol for 24 h. Matrigel-coated Boyden chambers were used to measure cell invasion. Western blot analyses were conducted to determine the levels of phosphorylated Akt (PAkt), β-catenin, MMP (matrix metalloproteinase)-9, and TIMP (tissue inhibitor of MMP)-1 protein. Zymography assays were used to detect MMP-9 activity. Our results show that PI3K does not arbitrate retinol- induced

dephosphorylation of Akt. In addition, Akt, but not PI3K, mediates the ability of retinol to decrease β-catenin protein levels. Also, expression of caPI3K and caAkt prohibits retinol from decreasing MMP-9 protein and activity, and increasing TIMP-1 protein levels. Finally, we show that the ability of retinol to decrease cell invasion was not diminished in cells expressing S37A mutant β-catenin. Thus, our data show retinol may inhibit the activity of PI3K and Akt individually to decrease colon cancer invasion and invasion-associated proteins independent of β-catenin.

INTRODUCTION

The five-year survival rate for colorectal cancer patients diminishes from 64% to 11% once distant metastases form (American Cancer Society 2009). Death due to colorectal cancer is generally not due to the initial tumor, but rather the metastases of the cancer, primarily to the liver (Scheele et al. 1995).

Retinoids have been shown to exhibit chemopreventative and chemotherapeutic properties in a several cancers (Tokar et al. 2006; Papi et al. 2007; Park et al. 2007; Liu et al. 2008; Lan et al. 2009; Papi et al. 2009). Most research in this area focuses on *all-trans* retinoic acid (ATRA), however the diet contains very little ATRA and (Vogel 1999) and as cancer progresses, tumors frequently become resistant to the actions of ATRA which diminishes the effectiveness of ATRA as cancer chemotherapy.

The retinoid vitamin A (retinol), present in animal-derived food sources, may prove to be an effective colon cancer therapy. Following absorption, colon cells are

primarily exposed to retinol (Harrison et al. 2001) and retinol is stored in the liver, the primary site of colorectal cancer metastasis (Vogel 1999). Serum retinol levels vary between 0.5-2 μM regardless of dietary levels (Smith et al. 1971). However, elevated intestinal lumen and hepatic concentrations of retinol have been attained by dietary vitamin A supplementation [for review see: (Loerch et al. 1979; Russell 2000)]. Since high levels of retinol can reach the colonocytes and the liver, where colon cancer primarily metastasizes, retinol may function as a powerful chemopreventive agent.

Previously our laboratory has found that dietary vitamin A supplementation decreases colorectal cancer cell metastasis in a nude mouse xenograft model (manuscript in preparation) and retinol decreases the invasion of ATRA-resistant human colon cancer cell via a novel retinoic acid receptor (RAR)-independent mechanism *in vitro* (Park et al. 2007). We have also demonstrated that retinol inhibits the activation of the multifaceted protein, PI3K (Park et al. 2008), and that PI3K mediates the ability of retinol to decrease cell invasion (manuscript in preparation, Fig. 1.3). In addition, retinol has been shown to reduce the activity and/or levels of several invasion-related proteins downstream of PI3K including Akt (manuscript in preparation, Fig. 1.4), β-catenin (Dillard et al. 2007) and matrix metalloproteinase (MMP)-9 (Park et al. 2007).

Elevated PI3K activity is associated with increased colon cancer cell invasion and metastasis (Keely et al. 1997; Shaw et al. 1997; Stephens et al. 2005). Activation of PI3K triggers a downstream signaling cascade resulting in the phosphorylation of several proteins including Akt (Franke et al. 1995), glycogen synthase kinase-3β (GSK3β) (Cross et al. 1995), and β-catenin (Cross et al. 1995; Weston et al. 2001), and expression of proteins such as MMPs (Brabletz et al. 1999; Qiu et al. 2004). Akt is a downstream

target of PI3K signaling that plays a critical role in cellular survival, proliferation, motility, and metastasis. Previous studies indicate that Akt over-expression is a frequent event in human colon cancer (Roy et al. 2002) that is correlated with increased invasion and metastasis (Itoh et al. 2002). To that end, we have shown that retinol decreases the phosphorylation of Akt and the ability of retinol to decrease cell invasion is mediated by Akt (manuscript in preparation, Fig. 1.4).

Akt activation phosphorylates GSK3β, an enzyme which facilitates the proteosomal degradation of β-catenin (Hart et al. 1998; Ikeda et al. 1998; Sakanaka et al. 1998). As Akt activity increases, GSK3β activity decreases via phosphorylation of the Ser9 residue. Inhibition of GSK3β activity causes increased cytosolic β-catenin concentrations. Excess cytosolic β-catenin migrates to the nucleus where β-catenin binds to T cell factor/ lymphoid enhancer factor (TCF/LEF) transcription factors, forming a potent transcription regulatory complex, inducing the transcription of various target genes regulating invasion and metastasis such as MMP-7 (Crawford et al. 1999). Previously, our lab has reported that retinol induces the proteasomal degradation of β-catenin via a retinoid-X-receptor (RXR)-dependent pathway in ATRA-resistant human colon cancer cells (Dillard et al. 2007; Dillard et al. 2008).

Activation of the PI3K/Akt pathway has also been associated with metastasis and vascular invasion through increased expression of MMP-9 in carcinomas (Chen et al. 2009). MMP-9 is a zinc-dependent extracellular gelatinase that promotes metastatic progression in colorectal cancer via highly specific degradation of extracellular matrix (ECM) proteins (Bernhard et al. 1994; Legrand et al. 1999; Bergers et al. 2000; McCawley et al. 2001; Huang et al. 2002). MMP-9 is over-expressed in colorectal

carcinomas (Heslin et al. 2001). Increased levels of MMP-9 expression in colorectal cancer compared with normal mucosa have been associated with significantly shorter disease-free and overall survival (Zeng et al. 1996). Reports have shown patients with colon carcinoma have a significant increase in levels of MMP-9 protein and up-regulation of MMP-9 transcription in tumor areas compared with noninvolved regions (Roeb et al. 2001; Herszenyi et al. 2008). Previously we have shown that retinol treatment reduced invasion of HCT-116 colon cancer cells by decreasing MMP mRNA levels as well as MMP-9 protein levels and activity (Park et al. 2007).

Tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) is a member of the TIMP family that inhibits MMP-9 activity (Mook et al. 2004) and has been shown to inhibit cell invasion and metastasis (Gomez et al. 1997). Retinoids have been shown to reduce cancer cell invasion via up-regulation TIMP-1 levels (Liu et al. 2003; Guruvayoorappan et al. 2008). Similarly, our laboratory has shown that retinol increases extracellular levels of TIMP-1 (Park et al. 2007).

Our preliminary data show that retinol decreases PI3K activity (Park et al. 2008), Akt phosphorylation (manuscript in preparation), β-catenin (Dillard et al. 2007), and MMP-9 levels (Park et al. 2007) and increases TIMP-1 in HCT-116 cells. Because PI3K and Akt activity regulate the effects of retinol on metastasis, and β-catenin is a downstream target of PI3K/Akt activation involved in the metastatic process, our objectives were to determine (1) if PI3K/Akt pathway mediates the inhibitory effects of retinol on downstream invasion-related proteins and (2) if β-catenin also regulates the ability of retinol to inhibit cell invasion *in vitro*. Specifically, we examined the ability of retinol to decrease cell invasion, Akt activation, β-catenin levels, MMP-9 activity and

protein levels, and increase TIMP-1 protein levels in HCT-116 human colon cancer cells (1) expressing two alleles of constitutively active (ca)PI3K, (2) stably transfected with caAkt, or (3) stably transfected with an S37A β-catenin mutant construct, resulting in over expression of degradation-resistant β-catenin.

MATERIALS AND METHODS

Tissue Culture

All HCT-116 human colorectal cancer cell lines were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics (1,000 U/ml penicillin and 1,000 μg/ml streptomycin) in a humidified atmosphere at 37°C with 5% CO_2.

HCT-116 cells expressing caPI3K were obtained from Dr. Bert Vogelstein of the Sidney Kimmel Comprehensive Cancer Center, Howard Hughes Medical Institute, and Johns Hopkins University Medical Institution (Baltimore, MA). These cells express to alleles of caPI3K and are referred to as "caPI3K" HCT-116 cells. HCT-116 cells expressing caPI3K were generated by homologous recombination of parental HCT-116 cells containing PI3K mutations in the kinase domain (H1047R alteration in exon 20) via an adeno-associated virus targeting system as described (Samuels et al. 2005). Parental HCT-116 cells, which express one wild-type and one constitutively active allele of PI3K were obtained from the ATCC (Manassas, VA).

In addition, parental HCT-116 cells, obtained from the ATCC (Manassas, VA) were stably transfected with caAkt plasmids (myr-Akt1) or vector control (pUSEamp+) constructs (Upstate Biotechnology, Billerica, MA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as per manufacturer's instructions. Transfectants were selected after culture with 500 μg/ml G418 in DMEM medium supplemented with 10% FBS and antibiotics for five weeks. Stable vector (pUSE) control transfections were confirmed by western blot analysis for neomycin phosphotransferase II (#06-747, Millipore, Billerica, MA). To confirm the presence of the transgene, stable caAkt transfectants were screened for phosphorylated Akt (#9275, Cell Signaling Technology, Danvers, MA), total Akt (#9272, Cell Signaling Technology, Danvers, MA), and myc tag (#06-549, Upstate Biotechnology, Billerica, MA). Selected caAkt cells contained more total Akt and phosphorylated Akt than vector (pUSE) transfected control cells and Akt phosphorylation was not decreased by retinol treatment.

Plasmids containing the S37A mutation of β-catenin and the empty vector (pcDNA3.1) were provided by Dr. Jones, Huntsman Cancer Institute, Utah. The S37A mutation results in the expression of a caβ-catenin–like phenotype by removing the N-terminal phosphorylation sites from β-catenin preventing the ubiquitination and subsequent proteasomeal degradation of β-catenin. Thus, transfection of cells with plasmids containing the S37A mutation results in higher levels of β-catenin protein. Parental HCT-116 cells were transfected with either the empty vector plasmid (pcDNA3.1) or the plasmid containing the S37A mutation. Transfectants were selected after culture with 500 μg/ml G418 in DMEM medium supplemented with 10% FBS and antibiotics for five weeks. The presence of the β-catenin S37A transgene was confirmed

by western blot analysis for Hemagglutinin Ab-1 (#RB-1438-P1, Neomarkers, Fremont, CA), β-catenin (#610153, Becton, Dickinson and Company, Franklin Lakes, NJ) and β-actin (#118K4827, Sigma Aldrich, St. Louis, MO). The presence of the pcDNA3.1 vector was confirmed by western blot analysis for neomycin phosphotransferase II (#06-747, Millipore, Billerica, MA). Selected stable cell lines over-expressing β-catenin (S37A) contained higher levels of β-catenin protein when compared to empty vector (pcDNA3.1) transfected control cells.

Zymography

MMP-9 activity was measured as previously described (Park et al. 2007). Parental or caPI3K HCT-116 cells, HCT-116 cells stably transfected with the caAkt plasmids (myr-Akt1) or vector control (pUSEamp+) constructs, and HCT-116 cells stably transfected with the S37A β-catenin or vector control (pcDNA3.1) constructs were plated at a density of 4 x 10^6 cells/150mm dish. Once attached, they were washed with phosphate buffered saline (PBS) twice and treated with 0 (ethanol vehicle control) or 10 μM retinol in serum free media for 24 h. Retinol stocks were dissolved in 100% ethanol and kept from light. MMP-9 activity was detected in serum free media concentrated via Amicon Ultra-15 Centrifugal Filters with a 30-kDa cutoff value (Millipore, Billerica, MA). Fifty μg of MMP-9 protein was separated using a 0.1% gelatin/8% non-denaturing polyacrylamide gel electrophoresis (PAGE) gel. Following electrophoresis, the gels were washed with 2.7% Triton X-100 for 30 minutes at room temperature and then incubated overnight with developing buffer (Invitrogen, Carlsbad, CA) at 37°C. Gels were stained

with 0.5% Coomassie Brilliant Blue-R250 and destained with 30% methanol and 10% acetic acid until visible white bands on blue background indicative of enzymatic activity were present. Enzymatic activity was analyzed for differences in groups via ImageQuant TL (GE Healthcare Life Sciences, Piscataway, NJ).

Western Blot Analysis

β-catenin, MMP-9, and extracellular TIMP-1 protein levels were measured as described previously (Dillard et al. 2007; Park et al. 2007). Parental or caPI3K HCT-116 cells, HCT-116 cells stably transfected with caAkt plasmids (myr-Akt1) or vector control (pUSEamp+) constructs, and HCT-116 cells stably transfected with S37A β-catenin or vector control (pcDNA3.1) constructs were plated and treated with retinol as described previously for zymography. Similarly, serum free media was concentrated using Amicon Ultra-15 Centrifugal Filters with a 30-kDa cutoff value (Millipore, Billerica, MA) 24 h after retinol treatment in order to isolate and measure extracellular MMP-9 and TIMP-1 proteins.

To determine if PI3K mediates the effects of retinol on Akt phosphorylation, parental of caPI3K HCT-116 cells were plated at 3 x 10^6 cells/100mm dish. Once attached, they were washed with PBS twice and treated with 0 (ethanol vehicle control) or 10 μM retinol in serum free media for 1 h.

Following retinol treatment, all cell lysates were harvested and intracellular protein was isolated using lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium

pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3OV_4, 1 mM phenylmethanesulphonyl fluoride (PMSF), and 1 μg/ml leupeptin). Protein concentrations were determined using the BioRad DC protein assay kit (Hercules, CA). 50 μg of protein was electrophoresed through 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS –PAGE) for phosphorylated Akt and β-catenin, 50 μg of protein through 8% SDS-PAGE for MMP-9, or 100 μg through 15% SDS-PAGE for TIMP-1. Following electrophoresis, the gels were transferred to nitrocellulose membranes. Membranes were blocked with 5% milk in Tris-Buffered Saline (TBST) (10 mM Tris, pH 8, 150 mM NaCl, and 0.05% Tween-20) for 1 h at room temperature before incubation with phosphorylated Akt (#9275, Cell Signaling Technology, Danvers, MA) and Akt (#9272, Cell Signaling Technology, Danvers, MA), β-catenin (#610153, Becton, Dickinson and Company, Franklin Lakes, NJ) and β-actin (#118K4827, Sigma Aldrich, St. Louis, MO), MMP-9 (#AB19016, Millipore, Billerica, MA), or TIMP-1 (sc-58435, Santa Cruz Biotechnology Inc., Santa Cruz, CA) primary antibodies at a 1:1000, 1:1000, 1:2000, 1:1000, or 1:100 dilution, respectively. Following subsequent incubation with corresponding secondary antibodies for 1 h at a 1:2000 dilution, immunoreactivity was detected using the Pierce Horseradish Peroxidase Super Signal West Pico Chemiluminescent Substrate kit (Rockford, IL). Developed chemiluminescent bands at the appropriate molecular weight were quantified using ImageQuant TL software (GE Healthcare Life Sciences, Piscataway, NJ).

Invasion Assay

To determine if β-catenin mediates the ability of retinol to reduce colon cancer cell invasion, Matrigel invasion assays were conducted as described (Park et al. 2007). HCT-116 cells stably transfected with S37A β-catenin or vector control (pcDNA3.1) constructs were serum starved for 48 h before seeding at a density of 1 x 10^5 cells per well on Matrigel-coated Boyden chambers. To measure cell invasion, the upper portion of the chambers contained 0 (ethanol vehicle control) or 10 μM retinol. An 8 μM pore-sized filter coated on the upper surface with Matrigel separated the cells from a lower chamber containing DMEM supplemented with 10% FBS which served as a chemoattractant. Cell invasion was measured after 24 h. All cells remaining in the upper portion of the chamber were removed. Cells that had migrated through the membrane were fixed in methanol prior to staining with Wrights Stain (Sigma-Aldrich, St. Louis, MO). The bottom of the filter was examined microscopically, and the number of stained cells present in 10 random fields of view counted. Invasion data was reported as % vehicle control. In addition, cell growth was measured in duplicate wells, lacking inserts, 24 h after treatment with and without retinol using a Coulter Counter. To normalize the data, the number of cells that had invaded through Matrigel was corrected for total cell number.

Statistical Analysis

Statistical tests were performed using Excel (Excel 2007 for Windows; Microsoft, Redmond, WA). All data were analyzed using two-tailed, Student's t-tests. All comparisons were made to vehicle control (0 μM) treated parental or empty vector

transfected HCT-116 cells. Differences were considered significant at $P < 0.05$. Values shown are expressed as mean ± SEM, n=3, unless otherwise indicated.

RESULTS

Expression of caPI3K does not block the ability of retinol to decrease β-catenin protein levels

As mentioned above expression of caPI3K blocks the ability of retinol to decrease cell invasion (manuscript in preparation, Fig. 1.3). Our laboratory has also shown that retinol, which is not a RXR ligand, reduced nuclear β-catenin protein levels in parental HCT-116 colon cancer cells by inducing β-catenin and RXRα binding resulting in their transport to the cytosol and subsequent RXR-mediated proteosomal degradation (Dillard et al. 2008). As previously mentioned, elevated β-catenin levels lead to increased cell invasion via the transcription of metastatic genes such as MMP-7 (Crawford et al. 1999).

To elucidate if PI3K mediates the ability of retinol to decrease β-catenin protein levels, we measured β-catenin protein levels in HCT-116 colon cancer cells expressing parental or caPI3K following 24 h of treatment with 0 (vehicle control) or 10 μM retinol. As shown in Fig. 2.1, retinol tended ($P = 0.1$) to decrease β-catenin levels in HCT-116 parental PI3K cells to 85.8 ± 7.0% of parental vehicle control, as expected. However, expression of caPI3K blocked the ability of retinol to decrease β-catenin protein levels. Specifically, expression of caPI3K significantly increased β-catenin protein levels to

110.7 ± 1.7% of parental vehicle-treated control and treatment with 10 μM retinol resulted in β-catenin protein levels 125.3 ± 16.5% of parental vehicle-treated control (Fig. 2.1). These data indicates that PI3K does not mediate the ability of retinol to decrease β-catenin protein levels.

Expression of caPI3K eliminates the ability of retinol to alter the activity and levels of the invasion-associated proteins, MMP-9 and TIMP-1 in conditioned media

MMPs facilitate cell invasion by digesting the extracellular matrix. Numerous studies have reported MMP-9 as a downstream target of PI3K/Akt activation and a key protein responsible for invasion in various carcinomas (Kim et al. 2001; Cheng et al. 2006; Arcaro et al. 2007; Chen et al. 2009). Previously, we showed that retinol treatment reduced the invasion of parental HCT-116 colon cancer cells by decreasing MMP-9 mRNA levels, MMP-9 protein levels and enzymatic activity (Park et al. 2007).

To examine if the ability of retinol to inhibit PI3K activity also conferred retinol's ability to decrease MMP-9 activity and protein levels, MMP-9 activity and protein levels were isolated from conditioned media obtained from HCT-116 cells expressing parental or caPI3K treated with 0 (vehicle control) or 10 μM retinol. As anticipated, treatment of parental HCT-116 cells with 10 μM retinol significantly reduced MMP-9 activity levels to 73.3 ± 4.7% and corresponding MMP-9 protein levels to 80.8 ± 6.2% of parental vehicle control (Fig. 2.2A and 2.2B, respectively). In contrast, retinol was unable to significantly decrease MMP-9 activity or protein levels in cells expressing caPI3K. Specifically, MMP-9 activity was reduced from 90.0 ± 7.2% to 79.0 ± 11.7% of vector control following retinol treatment. In particular, as seen in Fig. 2.2B, MMP-9 protein

levels did not decrease in cells treated with retinol expressing caPI3K.

TIMPs are endogenous protease inhibitors that regulate MMP activity and have been shown to inhibit cell invasion and metastasis (Gomez et al. 1997). Specifically, TIMP-1 inhibits MMP-9 activity (Mook et al. 2004). Previously we have shown that retinol treatment increased extracellular TIMP-1 protein levels (Park et al. 2007). In the current study, we examined the ability of retinol to increase TIMP-1 protein levels in the conditioned media obtained from HCT-116 cells expressing parental or caPI3K treated with 0 (vehicle control) or 10 μM retinol. As can be seen in Fig. 2.2C, expression of caPI3K prevented the capacity of retinol to increase TIMP-1 protein levels. Specifically, in parental cells, treatment with retinol tended to increase TIMP-1 protein levels to 121.7 ± 8.9% of parental vehicle control. On the contrary, TIMP-1 levels were not increased with retinol treatment in cells expressing caPI3K. Taken together, these data suggest that the ability of retinol to alter invasion-associated proteins, thereby inhibiting cell invasion, is regulated by PI3K.

Expression of caAkt prevents the ability of retinol to decrease β-catenin protein levels

Akt directly regulates the degradation of β-catenin by affecting the activity of GSK3β. Increased Akt activity results in increased cytosolic β-catenin concentrations and subsequent increased transcription of target genes regulating invasion and metastasis (Crawford et al. 1999). To determine if the ability of retinol to decrease β-catenin protein levels was mediated by the ability of retinol to inhibit Akt activity, we examined the β-catenin protein levels in HCT-116 cells stably transfected with caAkt or vector control plasmids treated with 0 (vehicle control) or 10 μM retinol for 24 h. In the vector

transfected cells, retinol tended ($P = 0.08$) to decrease β-catenin as expected (Fig. 2.3). Expression of caAkt blocked the ability of retinol to decrease β-catenin protein levels. As seen in Fig. 2.3, HCT-116 cells stably transfected with caAkt tended ($P = 0.07$) to contain higher levels of β-catenin protein than cells transfected with the empty vector. Specifically, cells expressing caAkt exhibited 120.9 ± 9.9% of vehicle-treated vector control ± SEM (Fig. 2.3). Retinol treatment did slightly reduce the amount of β-catenin protein in cells expressing caAkt to 102.1 ± 17.4% of the caAkt vehicle-treated control; however this decrease was not significant and may be due to the ability of retinol to inhibit endogenous Akt. These data show Akt mediates the ability of retinol to reduce β-catenin protein levels *in vitro*.

Expression of caAkt eliminates the ability of retinol to alter the activity and levels of the invasion-associated proteins, MMP-9 and TIMP-1 in conditioned media

Previous studies indicate that Akt over-expression is a frequent event in human colon cancer (Roy et al. 2002) that is correlated with increased invasion and metastasis (Itoh et al. 2002). MMP-9 is downstream of PI3K/Akt activation and our laboratory has shown that retinol decreases the activity of both Akt and MMP-9 (manuscript in preparation, Fig. 1.4; (Park et al. 2007; Chen et al. 2009). Therefore, we determined if the ability of retinol to inhibit Akt activation mediates the ability of retinol to to decrease MMP-9 activity and protein levels. We evaluated MMP-9 enzymatic activity and protein levels in conditioned media obtained from HCT-116 cells stably transfected with caAkt plasmids (myr-Akt1) or vector control (pUSEamp+) constructs treated with 0 (vehicle control) or 10 μM retinol. As can be seen in Figure 2.4A and B, retinol treatment

significantly reduced MMP-9 activity levels to 84.1 ± 4.7% and MMP-9 protein levels to of 94.9 ± 0.5% vehicle vector control in HCT-116 cells transfected with the empty vector control. Stably transfecting cells with caAkt increased MMP-9 activity levels when compared to vector-transfected cells (Fig. 2.4A). Expression of caAkt blocked the ability of retinol to decrease MMP-9 enzymatic activity and protein levels in conditioned media. In cells expressing caAkt, MMP-9 activity slightly increased to 128.7 ± 9.8 and 136.4 ± 2.3% of vehicle vector control (Fig. 2.4A) in cells treated with 0 and 10 μM retinol, respectively. MMP-9 protein levels were not significantly affected by caAkt expression, when compared to vector transfected cells. Importantly, retinol did not decrease MMP-9 protein levels in caAkt transfected cells (Fig. 2.4B). These data indicate that Akt regulates the ability of retinol to decrease MMP-9 activity and protein levels *in vitro*.

Expression of caAkt also eliminated the ability of retinol to increase TIMP-1 (Fig. 2.4C). In cells transfected with the empty vector, retinol tended to increase TIMP-1 protein levels, however in cells expressing caAkt, TIMP-1 protein levels were significantly reduced to approximately 84.3 ± 3.1 and 83.9 ± 3.4% of vehicle-treated vector control regardless of treatment (Fig. 2.4C). In summary, these data show that the ability of retinol to inhibit Akt activity mediates the effects of retinol on the invasion-associated proteins MMP-9 and TIMP-1.

The ability of retinol to decrease human colon cancer cell invasion is independent of β-catenin

Previously, we showed that retinol inhibits the cell invasion of HCT-116 cells in a dose-dependent manner (Park et al. 2007). In addition, we have found that retinol cannot

reduce the invasion of HCT-116 cells expressing caPI3K or caAkt (manuscript in preparation, Fig. 1.3 and Fig. 1.4), indicating that the ability of retinol to decrease PI3K and Akt activity mediates the retinol-induced inhibition of cell invasion. β-catenin is downstream of both PI3K and Akt and regulates cell invasion by inducing transcription of genes involved in metastasis (Crawford et al. 1999). We have previously shown that retinol reduces β-catenin protein levels by inducing its proteasomal degradation (Dillard et al. 2007; Park et al. 2007). In the present study, we stably transfected HCT-116 cells with a plasmid expressing the S37A β-catenin mutation or the corresponding empty vector plasmid (pcDNA3.1). As mentioned previously, this mutation results in β-catenin that is resistant to proteasomal degradation, increasing cellular β-catenin levels. Thus, we used the HCT-116 cells with S37A mutations to elucidate if β-catenin mediates the ability of retinol to reduce colon cancer cell invasion. Specifically, we examined the digestion and movement through Matrigel, a basement membrane-like protein matrix, of HCT-116 cells stably transfected with S37A β-catenin mutation or empty vector control (pcDNA3.1) plasmids following 24 h treatment with 0 (vehicle control) or 10 μM retinol. As expected, treatment of vector-transfected cells with 10 μM retinol significantly decreased cell invasion to 39.9 ± 7.6 % of vehicle-treated vector control cells (Fig. 2.5). Cells expressing the S37A β-catenin mutation exhibited a higher rate of invasion than the vector control cells when treated with the vehicle control. Specifically, transfection with S37A β-catenin increased the invasion of vehicle-treated cells to 194.8 ± 11.2% of vector vehicle control indicating that elevated β-catenin protein levels can increase the rate of cell invasion. However, elevated levels of β-catenin did not block the ability of retinol to decrease cell invasion. Treatment of cells with S37A β-catenin with 10 μM retinol

significantly decreased cell invasion to 154.1 ± 5.2 % of the vehicle-treated S37A β-catenin expressing cells (Fig. 2.5). Cell number was not affected after 24 h treatment with retinol in either cell type (data not shown). As can be seen in Fig. 2.5, retinol treatment reduces β-catenin in vector-transfected cells and additionally in cells transfected with S37A β-catenin. This data suggests that PI3K and Akt mediate the ability of retinol to decrease cell invasion independent of β-catenin. Because the results obtained from the β-catenin-over-expression invasion assays revealed that the effects of retinol on cell invasion are not arbitrated by β-catenin, we did not measure the invasion-related proteins MMP-9 and TIMP-1 with the S37A β-catenin cell line.

Expression of caPI3K does not block the ability of retinol to inhibit Akt activity

Previous work in our laboratory has shown that retinol decreases PI3K activity (Park et al. 2008) and the activation of Akt via phosphorylation (manuscript in preparation, Fig. 1.4A). We have also shown that the ability of retinol to decrease cell invasion is mediated by PI3K and Akt (manuscript in preparation; Fig. 1.3 and Fig. 1.4B). Therefore, we evaluated if the ability of retinol to decrease Akt activity was regulated by PI3K. Levels of phosphorylated Akt (P-Akt) were measured via western blot analysis in serum-starved HCT-116 colon cancer cells expressing parental or caPI3K following 1 h treatment with 10% FBS and 0 (vehicle control) or 10 μM retinol. As shown in Fig. 2.6, expression of caPI3K did not prevent retinol from decreasing Akt phosphorylation. Specifically, treatment of caPI3K cells with 10 μM retinol tended to decrease P-Akt protein levels to 62.5 ± 1.5% of the caPI3K vehicle-treated cells (Fig. 2.6). This result indicates that PI3K does not mediate the ability of retinol to decrease

Akt activity and suggests retinol may be directly interacting with Akt to decrease cell invasion.

DISCUSSION

Prior work in our laboratory has shown that retinol inhibits ATRA-resistant colon cancer cell invasion (Park et al. 2007), PI3K (Park et al. 2008), and Akt activity (manuscript in preparation, see Fig. 1.4A). We have also shown that expression of caPI3K eliminates the inhibitory effects of retinol on the invasion human colon cancer cells *in vitro* (manuscript in preparation, Fig. 1.3 and Fig. 1.4B). In the current study, we demonstrate that the ability of retinol to inhibit PI3K and Akt activity regulates the effects of retinol on the activity and protein levels of several downstream proteins involved in cell invasion such as β-catenin, MMP-9, and TIMP-1. Specifically, we observed that Akt, but not PI3K, mediates the ability of retinol to decrease β-catenin protein levels (Fig. 2.1 and 2.3). We also show that expression of caPI3K and caAkt blocked the ability of retinol to decrease MMP-9 protein and activity levels, and increase the levels of the MMP-9 inhibitor, TIMP-1 (Fig. 2.2 and 2.4). These data imply retinol inhibits PI3K and Akt signaling thereby reducing MMP-9 activity, increasing TIMP-1, and decreasing cell invasion. We also found that the ability of retinol to decrease cell invasion was not diminished by the expression of the S37A β-catenin mutation (Fig. 2.5), indicating that the ability of retinol to induce the degradation of β-catenin is not related to the ability of retinol to inhibit cell invasion. Finally, we show that expression of caPI3K

does not arbitrate the ability of retinol to reduce the phosphorylation and activity of Akt (Fig. 2.6), suggesting that the mechanism by which retinol inhibits invasion involves a reduction in Akt activity independent of PI3K.

Activation of the PI3K/Akt pathway contributes to tumor metastasis and resistance to chemotherapy (West et al. 2002). Previously we have shown that retinol treatment reduces PI3K activity in metastatic colon cancer cell lines *in vitro* (Park et al. 2008) and that retinol is unable to decrease the invasion of colon cancer cells expressing caPI3K (manuscript in preparation, Fig.1.3), indicating that the inhibitory effects of retinol on cell invasion is due to retinol-mediated inhibition of PI3K. Other studies have also shown that inhibition of PI3K activity by retinoids suppressed invasion and metastasis of cancer cells. For instance, ATRA decreased PI3K activity in vascular smooth muscle cells (Day et al. 2006) and decreases Akt activity, a target of PI3K, in the breast cancer cell lines MCF-7, SKBR3 and ZR-75 (del Rincon et al. 2003), and in the head and neck cancer cell line, SqCC/Y1 (Bastien et al. 2006). The effect of ATRA on PI3K activity has been shown to be mediated by RAR (Bastien et al. 2006; Day et al. 2006), however, the ability of retinol to inhibit PI3K is RAR-independent because (1) the cell line used in the current study lacks RARs (Sonneveld et al. 1998), (2) retinol is not a ligand for RAR, and (3) HCT-116 cells only produce a small amount of ATRA that does not activate RAR (Park et al. 2005). Therefore inhibition of PI3K-mediated invasion by retinol in ATRA-resistant HCT-116 colon cancer cells shows potential for a novel, effective anti-metastatic therapy for colon cancer.

PI3K signaling induces phosphorylation activation of Akt, which in turn modulates proteins involved in several cellular processes including proliferation, cell

survival, and motility (Stein 2001). Akt activation is required for suppression of cancer cell apoptosis and activation of tumor progression in human colorectal carcinoma (Itoh et al. 2002). In particular, elevated Akt activity is associated with increased cell invasion, tumor metastasis, and elevated cell survival in several cancers (Keely et al. 1997; Shaw et al. 1997; Stephens et al. 2005; Shukla et al. 2007; Pitt et al. 2009). Previously we have shown retinol decreases the phosphorylation activation of Akt in ATRA-resistant HCT-116 human colon cancer cells (manuscription in preparation) and the ability of retinol to decrease cell invasion is mediated by Akt (manuscript in preparation, Fig. 1.4). Similarly, application of tamibarotene, a synthetic selective retinoid that is more stable than ATRA, decreased the expression of Akt in human myeloma cells (Fukui et al. 2009). Because PI3K activates Akt, and retinol decreases the levels of both proteins, we determined if PI3K mediates the ability of retinol to inhibit Akt activity. Interestingly, we found that expression of caPI3K did not block the inhibitory effect of retinol on Akt. Similar results have been observed in other caPI3K models. For example, mutant *PI3CA*-bearing colon cancer cells treated with a PI3K inhibitor showed decreases in levels of phosphorylated Akt as seen in the current study (Guo et al. 2007). In addition, Vasudevan et al. 2009 suggests, after evaluating phosphor-protein profiling and genomic studies in *PIK3CA*-mutant cancer cell lines, PI3K may promote cancer through Akt-dependent and Akt-independent mechanisms (Vasudevan et al. 2009). Taken together, this data indicates that retinol may be independently acting upon PI3K and Akt to decrease cell invasion.

β-catenin is downstream of PI3K and Akt, and contributes to cell invasion by inducing transcription of genes involved in metastasis such as MMP-7 (Crawford et al.

1999). We have previously reported that retinol treatment decreases β-catenin and MMP-7 protein levels (Dillard et al. 2007; Park et al. 2007; Dillard et al. 2008). Similarly, retinoic "X" receptors (RXRs) have been shown to decrease β-catenin protein levels and subsequent β-catenin mediated gene transcription by inducing the proteasomal degradation of β-catenin in several colon cancer cell lines (Xiao et al. 2003; Dillard et al. 2008). Also, ATRA significantly inhibited the proliferation of glioma cells by altering the subcellular distribution of β-catenin and consequently increasing cytoplasmic β-catenin (Lu 2008). In addition, administration of ATRA, 9-*cis* retinoic acid, and 13-*cis* retinoic acid reduced MMP-7 expression and suppressed the invasiveness of several different colon cancer cells including the CHC-Y1, DLD-1, HT-29, BM314, CaR-1, and WiDr cells lines (Adachi et al. 2001). Here we demonstrate that HCT-116 human colon cancer cells expressing caPI3K exhibited decreased β-catenin protein levels following treatment with retinol as seen in parental HCT-116 cells (Fig. 2.1). This suggests that PI3K does not mediate the effects of retinol on β-catenin protein levels and β-catenin activated gene transcription (Fig. 2.1).

Previous studies have reported that MMP-9 is a downstream target of PI3K activation and a key protein responsible for invasion in various carcinomas (Cheng et al. 2006; Arcaro et al. 2007). Specifically, Chen et al (2009) reported that activated PI3K/Akt signaling pathway was associated with frequent intrahepatic metastasis and vascular invasion through up-regulation of MMP-9 expression in human hepatocellular carcinoma. Also, Chung et al (2004) found that hepatitis B virus X protein-induced expression of MMP-9 in hepatocellular carcinoma cells was reduced by inhibitors of the PI3K/Akt pathway. In the past we have shown that retinol inhibits PI3K activity (Park et

al. 2008), decreases MMP-9 mRNA, protein, and activity, and increases TIMP-1 protein levels (Park et al. 2007). Similarly, ATRA has been shown to decrease MMP-9 and increase tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) gene expression in murine lung alveolar carcinoma (Andela et al. 2004). In the current study, we determined if PI3K activity regulates the inhibitory capacity of retinol on the downstream invasion-associated proteins, MMP-9 and TIMP-1. Here we demonstrate that the ability of retinol to decrease MMP-9 activity and protein levels, and increase TIMP-1 protein levels was lost in cells expressing caPI3K (Fig. 2.2). Taken together, these data indicate that the ability of retinol to decrease cell invasion, and the effect of retinol on the invasion-associated proteins MMP-9 and TIMP-1 is mediated by PI3K activation.

Akt activation inhibits the activity of GSK3β, an enzyme which facilitates the proteasomal degradation of β-catenin (Hart et al. 1998; Ikeda et al. 1998; Sakanaka et al. 1998). As Akt activity increases, GSK3β-mediated degradation of β-catenin decreases. This results in an increase in stable β-catenin concentrations and subsequent β-catenin-TCF/LEF mediated gene transcription. Previous studies indicate that Akt over-expression/activation is highly correlated to invasion and metastasis in human colorectal cancer (Itoh et al. 2002; Johnson et al. 2010) and suggest that apoptosis inhibition during sporadic colon cancer carcinogenic process can be partially attributed to Akt (Roy et al. 2002). In addition, expression of dominant negative Akt resulted in inhibition of tumor growth and promotion of apoptosis in small cell lung cancer (Krystal et al. 2002). Similarly, genetic inactivation of the Akt isoforms, Akt1 and Akt2, resulted in a significant decrease in proliferation of HCT-116 or DLD1 colon cancer cells (Ericson et al. 2010). In the current study we show that expression of caAkt blocks the ability of

retinol to decrease β-catenin protein levels (Fig. 2.3). This data suggests inhibitory effects of retinol on β-catenin protein levels are mediated by Akt activity.

Mutations in the β-catenin degradation pathways are present in 70-80% of colorectal tumors (Pennisi 1998). β-catenin contributes to metastasis by transcriptional regulation of genes such as MMP-7. Our lab has reported that retinol reduced β-catenin protein and MMP-7 levels (Park et al. 2007; Dillard et al. 2008). In the current study, we evaluated if β-catenin regulates the effects of retinol on cell invasion. Here we show that HCT-116 human colon cancer cells expressing S37A β-catenin mutation exhibited decreased cell invasion following retinol treatment similar to parental control cells (Fig. 2.5) which indicates that retinol-induced inhibition of cell invasion is independent of β-catenin mediated gene transcription.

MMP-9 is over-expressed in colorectal carcinomas. High, active levels of MMP-9 promote metastatic progression in colorectal cancer via intravasation and extravasation (Bernhard et al. 1994; Legrand et al. 1999; Bergers et al. 2000; McCawley et al. 2001; Huang et al. 2002). As previously mentioned, retinoids have been shown to reduce invasion and metastasis by decreasing MMP protein levels or activity via inactivation of PI3K and Akt (Liu et al. 2003; Andela et al. 2004; Papi et al. 2007). Previously, we showed that retinol treatment reduced invasion of HCT-116 colon cancer cells by decreasing MMP-9 mRNA levels, MMP-9 protein levels, MMP-9 activity (Park et al. 2007), and Akt phosphorylation conferring the ability of retinol to inhibit cell invasion (manuscript in preparation, Fig. 1.4). In the current study we show that the ability of retinol to decrease MMP-9 activity and protein levels is dependent upon the capacity of retinol to inhibit Akt activity, as expression of caAkt blocks the decrease in MMP-9

protein and activity previously seen in parental ATRA-resistant HCT-116 human colon cancer cells (Fig. 2.4). These reports and our current study support the involvement of PI3K/Akt signaling in transcriptional regulation of MMP-9 and elucidate the part of the pathway by which retinol decreases cell invasion.

TIMPs are endogenous protease inhibitors that regulate MMP activity. TIMPs have been shown to inhibit cell invasion *in vitro*, tumorigenesis, metastasis *in vivo*, angiogenesis, and reduce tumor cell growth (Gomez et al. 1997). TIMP-1 prevents the activity of most MMPs (Mook et al. 2004). Specifically, TIMP-1 inhibits MMP-9 activity by binding to pro-MMP-9 which prevents the conversion of pro-MMP-9 to active MMP-9 (Weinzweig et al. 2003). Previously it has been shown that ATRA downregulates MMP-9 by up-regulating TIMP-1 expression (Dutta et al. 2010). Our laboratory has also shown that treatment with retinol increases extracellular TIMP-1 protein levels (Park et al. 2007). In the current study we show that expression of caPI3K or caAkt blocked the ability of retinol to increase TIMP-1 protein levels which supports current literature and suggests PI3K and Akt mediate the effects of retinol on TIMP-1 protein levels (Fig. 2.8). The exact link between PI3K/Akt and TIMP-1 expression is unclear. Several studies have implicated the involvement of the signaling protein, transforming growth factor-β1 (TGF-β1) in the regulation of TIMP-1 expression in normal and cancerous tissues (Sehgal et al. 1999; Hall et al. 2003; Kwak et al. 2006; Offenberg et al. 2008). However, the direction of regulation (i.e. increase or decrease in expression) caused by TGF-β1 is not well understood and depends on the type of cell and stage of malignancy. Recently, Bian et al. 2009 reported that TGF-β1 signaling and PI3K/Akt pathway cross-talk in mice with head and neck carcinogenesis (Bian et al.

2009) which implicates a possible link between PI3K/Akt and TIMP-1 expression. Further analysis of this association is warranted.

Our results support the hypothesis that inhibition of PI3K and Akt by retinol mediates the ability of retinol to reduce colon cancer cell invasion via regulation of MMP-9 and TIMP-1 protein levels. However, the link between PI3K and Akt inactivation and subsequent alterations in MMP-9 and TIMP-1 levels remain unclear. Retinol may be inhibiting cell invasion by decreasing MMP-9 activity via an alternative PI3K/Akt pathway involving NF-κB. MMP-9 expression is induced via NF-κB transcriptional activation. NF-κB is a potent transcription factor that is responsible for the expression of numerous genes implicated in a wide array of cellular processes including inflammation, cell survival, proliferation, immune responses, angiogenesis, invasion, and metastasis [For a review see: (Lee et al. 2007)]. NF-κB activity is absolutely necessary for MMP-9 expression (Bond et al. 1998; Bauerle et al. 2010). Under inactive conditions, NF-κB is located in the cytoplasm bound to an inhibitory protein known as IκB. In stimulated cells, IκB degradation is induced by a multi-subunit IKK complex composed of two catalytic subunits, IKKα and IKKβ (Romashkova et al. 1999). IKK phosphorylation of IκB on the Ser 32 and Ser 36 residues results in IκB ubiquitination and proteasomal degradation (Romashkova et al. 1999). Removal of IκB liberates NF-κB which allows translocation to the nucleus and NF-κB transcription activation of target genes including MMP-9 (Bond et al. 1998).

NF-κB activity has been shown to be induced by Akt and PI3K mediated phosphorylation (Ozes et al. 1999; Romashkova et al. 1999). Specifically, Akt activates NF-κB activity by phosphorylating the IKKα subunit of the IKK complex, which in turn

allows IKK to phosphorylate and activate the p65 subunit of NF-κB (Haller et al. 2002) and induce MMP-9 expression. In addition, Akt has recently been shown to stimulate NF-κB activity indirectly through an IKKα/β-catenin mediated process in colon cancer cells (Agarwal et al. 2005). IKKα activity mediated by Akt activation not only directly stimulates NF-κB gene transcription, but also can indirectly up-regulate β-catenin-dependent gene transcription (Lamberti et al. 2001; Albanese et al. 2003). The Akt/ IKKα signaling pathway may therefore be a key contributor to colon cancer progression via deregulation of transcription of genes involved in tumor metastasis such as MMP-9. Future experiments should investigate the role of retinol in inhibiting this Akt/IKK signaling activities.

Recently, Dutta et al. 2010 reported that ATRA reduced MMP-9 expression in MDA-MB-231 human breast cancer cells via regulation of PI3K and NF-κB signaling pathways. We have shown that PI3K mediates the ability of retinol to decrease cell invasion (Fig. 1.3). PI3K may contribute to the activation of NF-κB mediated MMP-9 expression through a 3'-phosphoinositide-dependent kinase-1 (PDK-1)/protein kinase C (PKC) dependent pathway.

PDK-1 is a serine/threonine protein kinase downstream of PI3K known to be involved in activation of several isoforms of PKC including the conventional (cPKC), novel (nPKC), and atypical (aPKC) types (Storz et al. 2002). Specifically, it has been reported that phosphorylation and activation of the atypical isoforms, PKCdelta and PKCzeta, are stimulated by PDK-1 and inhibited by wortmannin, which suggests the activity of these proteins is dependent on PI3K activity (Storz et al. 2002). Therefore it is plausible to hypothesize that PI3K activates PDK-1-dependent phosphorylation activation

of PKC, which in turn positively regulates NF-κB-mediated gene expression of proteins involved in metastasis such as MMP-9.

In particular, protein kinase C-zeta (PKCzeta) or protein kinase C-delta (PKCdelta) have been shown to induce NF-κB gene transcription by phosphorylating the IKKβ subunit of the IKK complex and thereby activating NFκB mediated transcription (Hirai et al. 2003). Several studies have supported the roles of PKCdelta and PKC zeta in activating cell proliferation, migration, and metastatic pathways in many cancers (Cerda et al. 2006; Mertens-Walker et al. 2010) (Lai et al. 2010). The current literature suggests that PKCs act as important messengers for the transcription regulation of MMP genes. For example, it has been shown that application of PKCdelta inhibitors reduced the invasion of MCF-7 human breast cancer cells by decreasing MMP-9 secretion via PKCdelta/AP-1/NF-κB mediated pathway (Park et al. 2009). This evidence gives rise to investigate the involvement of PKC in metastasis of colon cancer and the possible role of retinol in inhibiting the actions of this protein to modulate cell invasion.

In many types of cancer, NF-κB is constitutively active, nonetheless previous literature has reported that inhibition of NF-κB activity reduces MMP-9 activity and expression and metastasis in several cancers, including colorectal cancer (Huang et al. 2001; Shih et al. 2007; Park et al. 2009; Lu et al. 2010; Yang et al. 2010). NF-κB is not a focus of the current study; however due to the reported regulation of MMP-9 and consequential invasion via PI3K and Akt activation, it presents an area of future interest examining possible effects of retinol on NF-κB activity.

The current study shows that PI3K and Akt mediate the ability of retinol to decrease MMP-9 and increase TIMP- levels, and consequential cell invasion,

independent of β-catenin. These results suggest that PI3K and Akt may independently modulate MMP-9 expression. We propose a new mechanism of colon cancer metastasis involving PI3K, Akt, and NF-κB pathways that result in MMP-9 expression and subsequent tumor progression. Future studies are warranted to examine the hypothetical model proposed in Fig. 2.7 and determine if the mechanism by which retinol reduces colon cancer metastasis involves direct or indirect interaction with these downstream invasion-related proteins.

ACKNOWLEDGEMENTS

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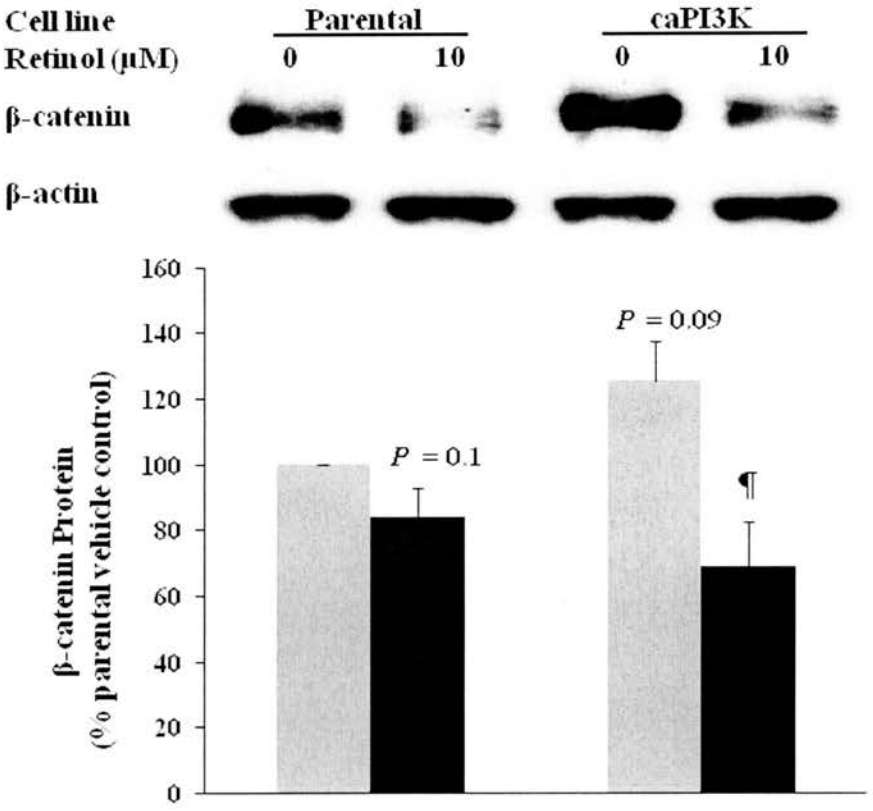
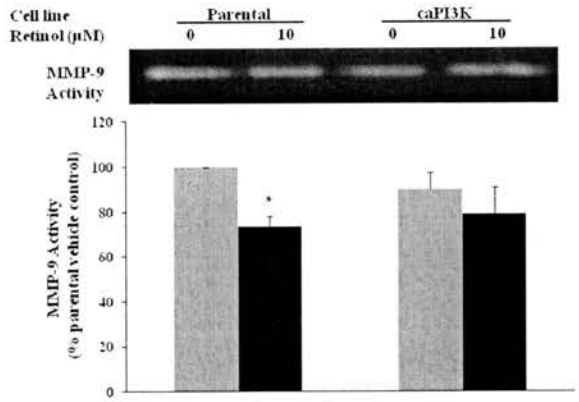


Figure 2.1. Expression of caPI3K does not block the ability of retinol to decrease β-catenin protein levels. Total intracellular protein was isolated from parental or caPI3K-expressing HCT-116 cells treated with 0 (ethanol vehicle control) or 10 μM retinol for 24 h. β-catenin protein was detected using β-catenin antibody as described in Materials and Methods. β-Actin was used as an internal loading control. Data are reported as mean ± SEM; n=3. *Significantly different from parental control ($P < 0.05$). ¶Significantly different from caPI3K vehicle control ($P < 0.05$).

A



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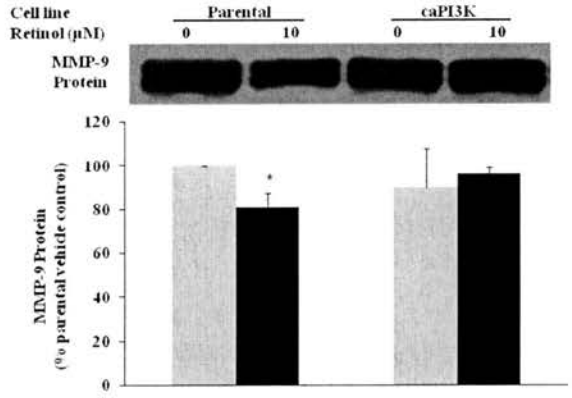

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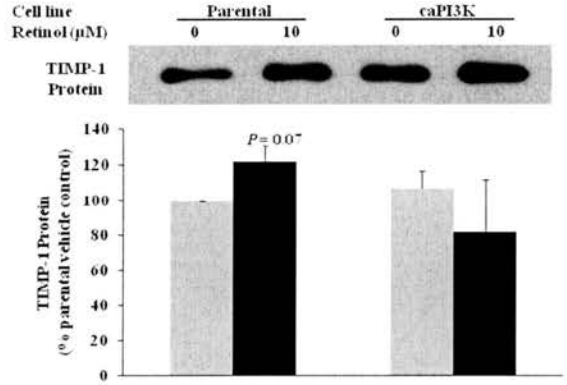
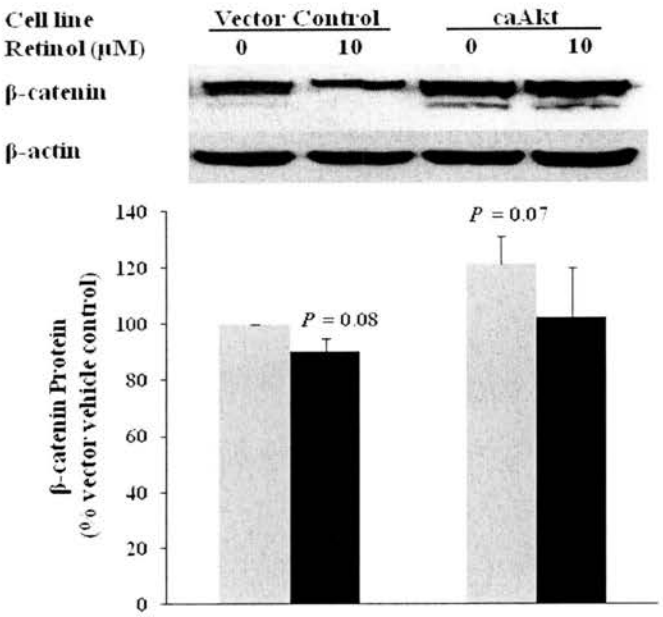


Figure 2.2. Expression of caPI3K eliminates the ability of retinol to alter the activity and levels of the invasion-associated proteins, MMP-9 and TIMP-1 in conditioned media. Parental or caPI3K-expressing HCT-116 cells were treated with 0 (ethanol vehicle control) or 10 μM retinol for 24h. (**A**) Gelatin zymogram displaying the active form of MMP-9 (92 kDa). Western blot displaying active MMP-9 (**B**) and TIMP-1 (**C**) protein levels in serum-free conditioned media. Data are reported as mean ± SEM; n=3. *Significantly different from parental vehicle control ($P < 0.05$).

A



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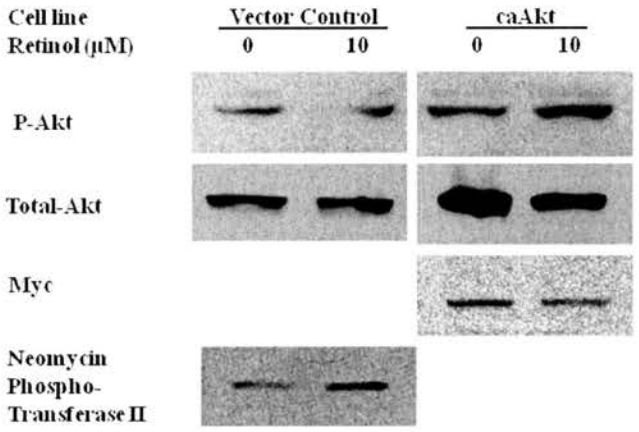


Figure 2.3. Expression of caAkt prevents the ability of retinol to decrease β-catenin protein levels. (**A**) Total intracellular protein was isolated from HCT-116 cells stably transfected with caAkt plasmids (myr-Akt1) or vector control (pUSEamp+) constructs treated with 0 (ethanol vehicle control) or 10 μM retinol for 24 h. β-catenin protein was detected using β-catenin antibody as described in Materials and Methods. β-Actin was used as an internal loading control. Data are reported as mean ± SEM; n=3. *Significantly different from vector vehicle control ($P < 0.05$). (**B**) Stable vector (pUSE) control transfections were confirmed by western blot analysis for neomycin phosphotransferase II (Millipore #06-747). The presence of the caAkt transgene was confirmed by western blot analysis for phosphorylated Akt (Cell Signaling #9275), total Akt (Cell Signaling #9272), and myc tag (Upstate Biotechnology #06-549). One representative western blot is shown.

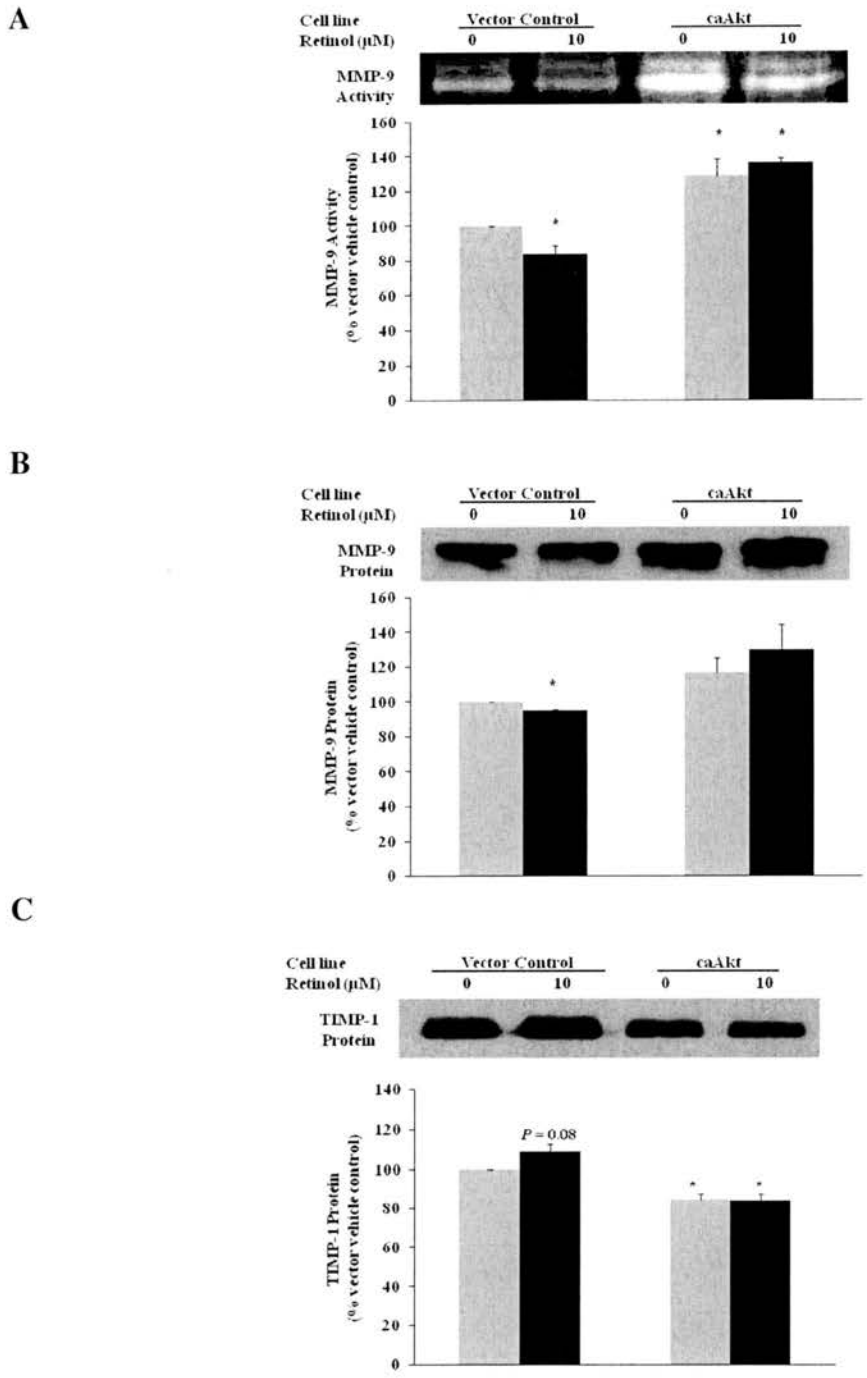
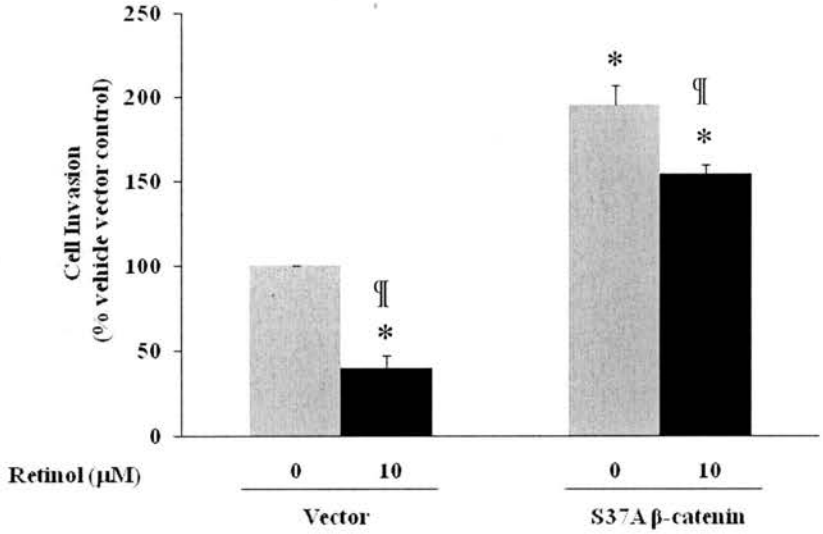


Figure 2.4. Expression of caAkt eliminates the ability of retinol to alter the activity and levels of the invasion-associated proteins, MMP-9 and TIMP-1 in conditioned media. HCT-116 cells stably transfected with caAkt plasmids (myr-Akt1) or vector control (pUSEamp+) construct were treated with 0 (ethanol vehicle control) or 10 μM retinol for 24 h. (**A**) Gelatin zymogram displaying active MMP-9 (92 kDa). Western blot displaying MMP-9 (**B**) and TIMP-1 (**C**) protein levels in serum-free conditioned media. Data are reported as mean ± SEM; n=3. *Significantly different from vector vehicle control ($P < 0.05$). Presence of the transgene is shown in Fig. 2.3B.

A



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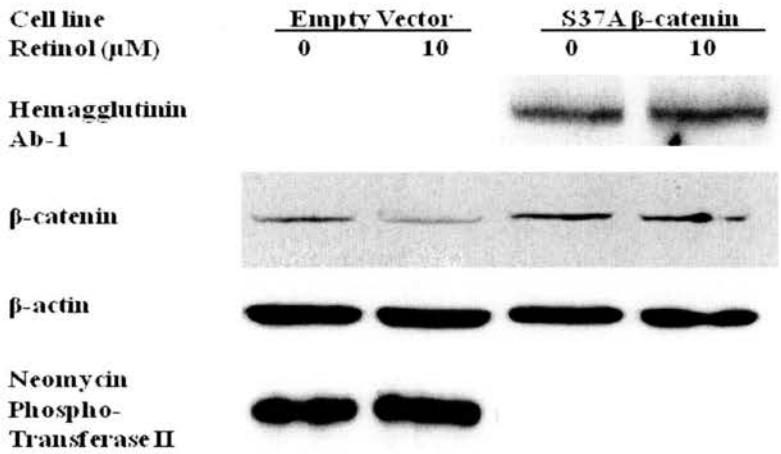

Figure 2.5. The ability of retinol to decrease human colon cancer cell invasion is independent of β-catenin. (**A**) HCT-116 cells stably transfected with S37A β-catenin mutation or empty vector control (pcDNA 3.1) were serum starved for 48 h before seeding at a density of 1 x 10^5 cells per well on Matrigel-coated Boyden chambers. The upper portion of the chambers contained 0 (ethanol vehicle control) or 10 μM retinol. The lower chamber contained 10% FBS which served as a chemoattractant. Cell invasion was measured after 24 h by staining with Wrights Stain as described in Materials and Methods. All data are reported as mean ± SEM; n=3. *Significantly different from vector vehicle control ($P < 0.05$). ¶Significantly different from S37A β-catenin vehicle control ($P < 0.05$). (**B**) Stable vector control (pcDNA3.1) transfections were confirmed by western blot analysis for neomycin phosphotransferase II. The presence of the S37A β-catenin mutation was confirmed by western blot analysis for hemaglutinin Ab-1, β-catenin, and β-actin. One representative western blot is shown.

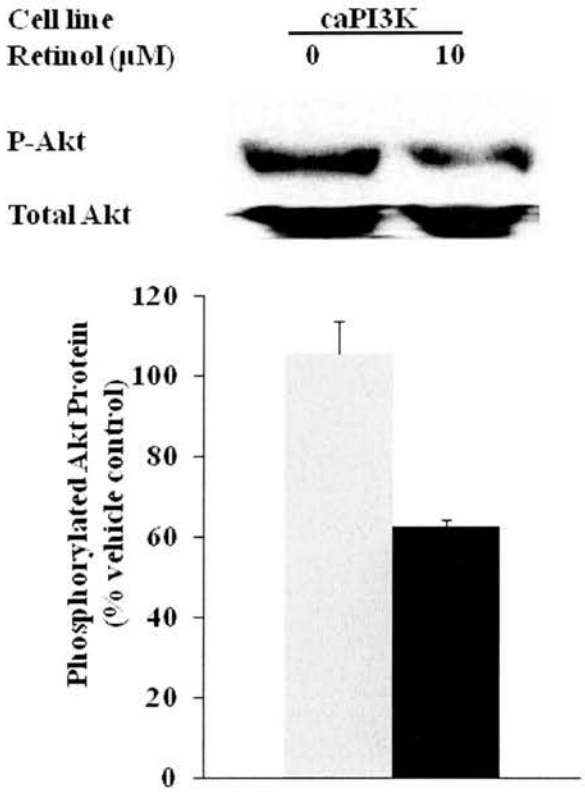

Figure 2.6. Expression of caPI3K does not block the ability of retinol to inhibit Akt activity. Total intracellular protein was isolated from parental or caPI3K-expressing HCT-116 cells treated with 0 (ethanol vehicle control) or 10 μM retinol for 24 h. Phosphorylated Akt protein was detected using P-Akt (#9275, Cell Signaling Technology) antibody as described in Materials and Methods. Total Akt protein was used as an internal loading control. Data are reported as mean ± SEM; n=2.

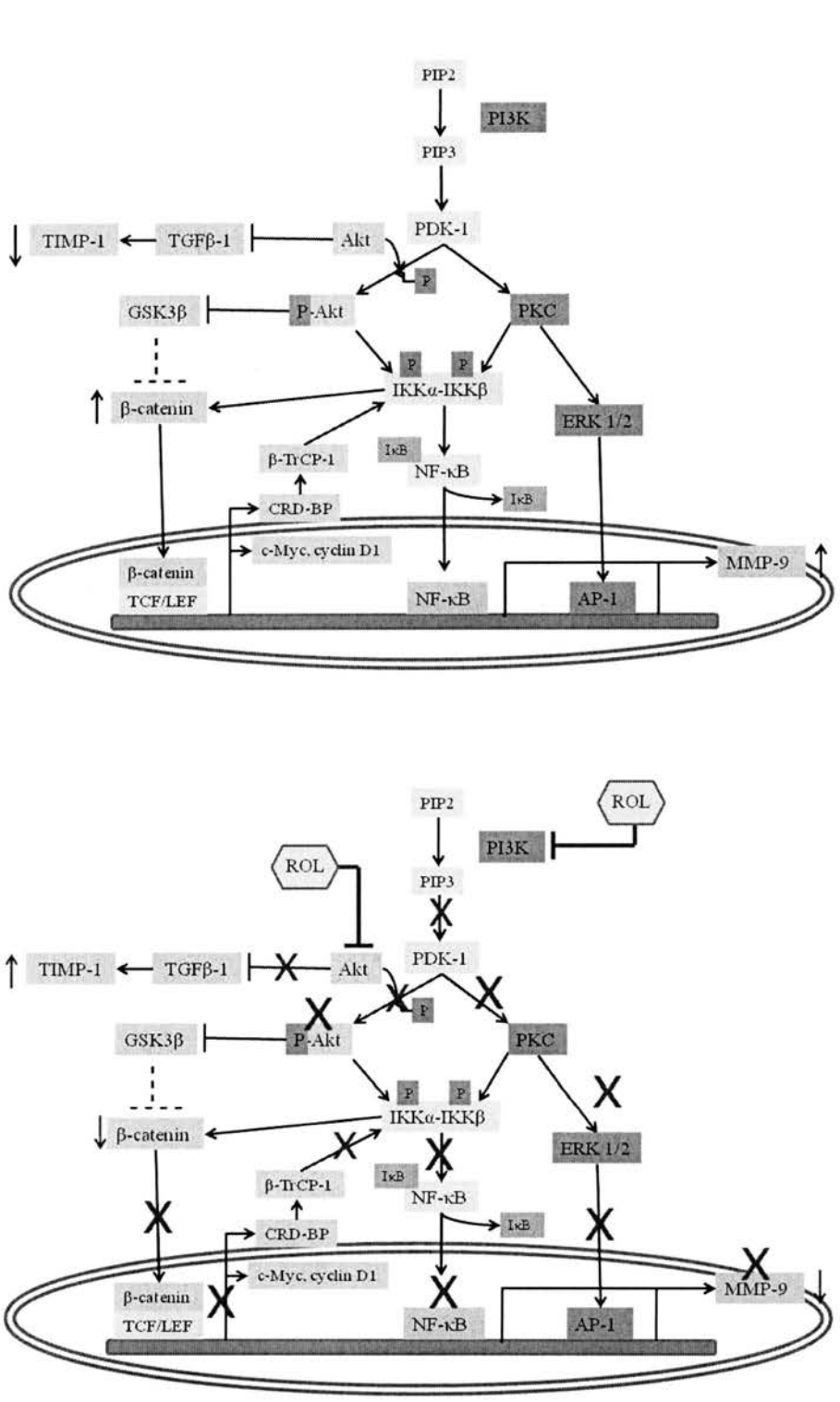

Fig 2.7. Potential pathway of colon cancer metastasis mediated by retinol. Hypothetical pathway of colon cancer metastasis involving PI3K and Akt activity (**A**). Possible effects of retinol on proteins involved in metastasis (**B**). Arrows indicate an increase or decrease in expression; "X" indicates the function or signal is inhibited.

III. PHOSPHATIDYLINOSITOL 3-KINASE MEDIATES THE ABILITY OF DIETARY VITAMIN A TO DECREASE THE INCIDENCE OF HEPATIC METASTASES OF COLORECTAL CANCER IN A MOUSE XENOGRAFT MODEL

ABSTRACT

Previous work in our laboratory has shown that vitamin A supplementation decreases colorectal cancer metastasis in a mouse xenograft model. In addition, we have shown that retinol inhibits phosphatidylinositol 3-kinase (PI3K) activity *in vitro*. The objective of the current study was to determine if PI3K mediates the ability of dietary vitamin A to decrease the metastasis of HCT-116 human colon cancer cells in a mouse xenograft model. ATRA-resistant HCT-116 cells expressing parental or constitutively active (ca) PI3K were intrasplenically injected into female BALB/cAnNCr-nu/nu nude mice to establish a splenic tumor which then shed cells that metastasized to the liver. Vitamin A, in the form of retinyl palmitate, was supplemented in the diet at six ascending levels (2,400 (control), 12,000, 25,000, and 100,000 IU/kg diet) one month prior to tumor cell injection and for 5 weeks after injection, until sacrifice. Liver tumor incidence, multiplicity, and tumor size were assessed. Dietary supplementation of mice injected

with parental HCT-116 cells with 100,000 IU vitamin A/kg diet decreased liver tumor incidence. Specifically, 100,000 IU vitamin A/kg diet resulted in a metastatic incidence to 56.8% that of the control dietary group injected with parental HCT-116 cells. Consumption of 12,000, 25,000, and 100,000 IU vitamin A/kg diets in the group injected with caPI3K HCT-116 cells exhibited an incidence 146.6, 140, and 110% that of the caPI3K control group. Metastatic multiplicity and size were not affected by dietary vitamin A supplementation in mice injected with either the parental or caPI3K-expressing HCT-116 cells. These data demonstrate that PI3K mediates the ability of vitamin A supplementation to decrease liver metastasis incidence *in vivo,* confirming our *in vitro* results and revealing a piece of the mechanism by which vitamin A inhibits metastasis.

INTRODUCTION

Colorectal cancer is the third most common cancer and second most common cause of death due to cancer in the United States (A.C.S. 2009). The five-year survival rate for colorectal cancer patients diminishes from 64% to 11% once distant metastases form. Death due to colorectal cancer is generally not due to the primary tumor, but rather the metastases of the cancer, primarily to the liver (Scheele et al. 1995).

The retinoids, a group of compounds consisting of vitamin A (retinol), its natural metabolites (ATRA), and several synthetic compounds, have been shown to inhibit metastasis in a variety of model systems (Liu et al. 2003; Andela et al. 2004; Heukamp et al. 2005; Papi et al. 2007; Park et al. 2007; Liu et al. 2008; Lan et al. 2009; Papi et al. 2009). Although most research in this area focuses on *all-trans* retinoic acid (ATRA),

the diet contains very little ATRA and serum ATRA concentrations are low (1-14 nM) (Vogel 1999). In addition, as cancer progresses, tumors frequently become resistant the inhibitory effects of ATRA. ATRA resistance is caused by a defect in retinoic acid receptor (RAR) induction in response to ATRA (Sonneveld et al. 1998; Nicke et al. 1999; Lee et al. 2000) due to methylation of the RARE in the RAR's promoter region. Preformed dietary vitamin A is consumed as retinol and retinyl esters from animal-derived food sources. Retinyl esters are cleaved within the intestinal lumen to yield retinol. Upon absorption from the gut, retinol is esterified, forming retinyl esters that are incorporated into chylomicrons, bound to retinol-binding proteins (RBPs), and sent to the liver, the main retinol storage site (Vogel 1999). In addition to the retinol present in the intestinal lumen, retinol can reach colonocytes via circulation bound to RBPs or as free retinol (Harrison et al. 2001).

Serum retinol levels vary between 0.5-2 μM regardless of dietary levels (Smith et al. 1971). However, elevated intestinal lumen and hepatic concentrations of retinol have been attained by dietary vitamin A supplementation [for review see: (Loerch et al. 1979; Russell 2000; Schmidt et al. 2003; Garcia et al. 2005)]. Specifically, mice consuming milk from dams fed a diet supplemented with 589,091 IU vitamin A/kg until 21 days of age and then the supplemented diet itself until 65 days of age reported retinol concentrations as high as 90.8 μM (26.0 $\pm$ 3.2 μg/g) (Garcia et al. 2005). In addition, consumption of 6,000 IU/kg diet by rats resulted in a hepatic retinol concentration of 6.4 μM (Schmidt et al. 2003). Hence dietary vitamin A supplementation can enhance retinol concentrations in the colon and liver, making retinol a potential inhibitor of colorectal cancer metastasis. To that end, our laboratory has found that dietary vitamin A

supplementation decreases colorectal cancer cell metastasis in a nude mouse xenograft model (manuscript in preparation). We have also shown that retinol decreases the invasion of ATRA-resistant human colon cancer cell via a novel retinoic acid receptor (RAR)-independent mechanism *in vitro* (Park et al. 2007).

To determine the mechanism by which vitamin inhibits colon cancer metastasis, our laboratory has also investigated the effects of retinol on invasion-related proteins. In particular, we have examined the multi-faceted protein phosphatidylinositol 3-kinase (PI3K) which plays a key role in the regulation of many cellular processes including proliferation, cell survival, carbohydrate metabolism, and motility (Stein 2001). PI3K is somatically mutated in over 25% of colorectal tumors and amplification of genomic regions containing PI3K genes has been reported (Samuels et al. 2005). Activation of PI3K is associated with an invasive phenotype in human colon cancer (Stephens et al. 2005). Elevated PI3K activity triggers a downstream signaling cascade resulting in the over-expression of several proteins involved in cell proliferation (e.g. cyclin D1 and c-Myc) and metastasis (e.g. matrix metalloproteinase(MMP)-7 and MMP-9) which has been associated with amplified colon cancer cell invasion and metastasis (Keely et al. 1997; Shaw et al. 1997; Stephens et al. 2005).

Previous nude mouse xenograft models of hepatic metastasis have shown a relationship between metastatic progression and PI3K levels. For example, nude rats injected via mesenteric vein with HT-29d colon cancer cells, select highly metastatic variants derived from HT-29 cells, exhibited a higher expression of PI3K compared to non- and low metastatic cells lines, suggesting that the metastatic potential of colorectal carcinoma may positively correlate with the levels of PI3K and the invasive stage of

colon cancer (Wang et al. 2003). Rychahou et al. 2006 reported that nude mice intrasplenically injected with HT-29 colon cancer cells and PI3K-specific siRNA treatment resulted in growth inhibition of hepatic tumor metastases (Rychahou et al. 2006). In addition, HCT-116 colon cancer cells expressing constitutively active PI3K displayed enhanced liver metastatic potential compared to HCT-116 wild-type PI3K colon cancer cells in an *in vivo* orthotopic BALB/c nude mouse model (Guo et al. 2007). Recently, orthotopic implantation into nude mice of HCT-116 colon cancer cells expressing Ron kinase-knockout demonstrated significantly decreased distant metastases and activated PI3K compared to control animals, affirming the role of PI3K in colon cancer metastasis (Wang et al. 2009). To that end, our laboratory has previously reported that retinol inhibits PI3K activity (Park et al. 2008) and that PI3K mediates the inhibitory effects of retinol on cell invasion *in vitro* (manuscript in preparation, see Fig. 1.3). These data compelled us to investigate if the role of retinol in prohibiting colon cancer metastasis is arbitrated by PI3K activity.

Our preliminary data show that retinol decreases HCT-116 colon cancer cell invasion independent of ATRA and RARs (Park et al. 2007), PI3K activity (Park et al. 2008), and PI3K regulates the ability of retinol to decrease HCT-116 colon cancer cell invasion (manuscript in preparation, see Fig. 1.3) in vitro. In addition, vitamin A supplementation decreases colorectal cancer metastasis *in vivo*. Because PI3K regulates metastasis, the objective of the present study was to determine if PI3K mediates the inhibitory effects of dietary vitamin A supplementation on the hepatic metastases of colon cancer tumor cells *in vivo*.

MATERIALS AND METHODS

Tissue Culture

Parental HCT-116 cells, which express one wild-type and one constitutively active allele of PI3K were obtained from the American Type Culture Collection (Manassas, VA). HCT-116 human colon cancer cells expressing caPI3K were obtained from Dr. Bert Vogelstein of the Sidney Kimmel Comprehensive Cancer Center, Howard Hughes Medical Institute, and Johns Hopkins University Medical Institution (Baltimore, MA). HCT-116 cells expressing constitutively active PI3K were generated by homologous recombination of parental HCT-116 cells containing PI3K mutations in the kinase domain (H1047R alteration in exon 20) via an adeno-associated virus targeting system as described (Samuels et al. 2005). HCT-116 cells expressing two alleles of constitutively active PI3K will be referred to as "caPI3K" cells. All HCT-116 cells were grown in McCoy's medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (1,000 U/ml penicillin and 1,000 μg/ml streptomycin) in a humidified atmosphere at 37°C with 5% CO_2.

Xenograft Model

This animal study was performed in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals under Animal Welfare Assurance number A4107-01, University of Texas Institutional Animal Care and Use Committee (IACUC) protocol number 08062501, and reciprocal Texas State University IACUC protocol number 0923-0625-20.

To determine if PI3K mediates the ability of retinol to inhibit colon cancer cell metastasis *in vivo,* 96 BALB/cAnNCr-nu/nu mice aged 6 to 8 weeks were randomly assigned to diets containing increasing amounts of vitamin A. Twenty-four mice were assigned to each dietary group. All diets (Research Diets, Inc., New Brunswick, NJ) were irradiated, purified, and pelleted containing 2,400 (control), 12,000, 25,000, or 100,000 IU of vitamin A as retinyl palmitate per kg of diet (See Table 3.1 for diet composition). Diets and water were provided fresh every other day and consumed *ad libitum.* Feed intake was recorded every other day and body weight weekly. All diets were stored refrigerated and light exposure was avoided.

The vitamin A content of the control diet was based on the dietary vitamin A requirement for mice from the National Research Council (National Research Council (U.S.). Subcommittee on Laboratory Animal Nutrition. 1995). The highest supplementation was chosen based on previous studies (Weinzweig et al. 2003; Delage et al. 2004) and on data obtained from an *in vivo* experiment performed in our laboratory (Park and Lane, in preparation). Due to the possibility of vitamin A toxicity, the diets containing the 12,000, 25,000, and 100,000 IU vitamin A/kg were assessed to determine if a lower level of vitamin A supplementation would show the same effect as 200,000 IU/kg vitamin A supplementation. Consumption of the control and vitamin A-supplemented diets began one month prior to intrasplenic colon cancer cell injection and continued until sacrifice. Throughout the period of supplementation, the mice were checked 3x per week for cutaneous signs of vitamin A toxicity and any symptoms were reported.

One month after being placed on their respective diets, mice were intrasplenically injected with colorectal tumor cells. Specifically, 1 x 10^6 parental HCT-116 or caPI3K-HCT-116 cells in a single-cell suspension in Hank's Balanced Salt Solution (HBSS) without calcium and magnesium were injected into the spleen of each mouse. Within each dietary group 12 mice were injected with parental HCT-116 cells and 12 injected with caPI3K-HCT-116 cells.

For each group, the viability of the parental or caPI3K HCT-116 cell suspension was evaluated before and after surgery via trypan blue dye exclusion assay. Cell viability following surgery was 97% for both the parental and caPI3K HCT-116 cells.

Five weeks after tumor cell injection, all mice were euthanized. Blood was obtained via cardiac puncture. The liver and spleen of each mouse were excised, divided in two and fixed in 10% formalin or frozen in liquid nitrogen for histology and high pressure liquid chromatographic analysis of retinoid content, respectively. Mice were fasted for 12 h, prior to sacrifice, to eliminate possible postprandial effects on serum or liver retinoid levels.

Hepatic metastatic tumor incidence and multiplicity were assessed. Additionally, the number of visible liver metastases (white nodules) and the diameter of each nodule observed were determined. Visible hepatic tumors were classified according to their size and labeled as: small, medium, and large metastasis. "Small metastasis" was defined as a tumor composed of small colonies of neoplastic cells visible to the naked eye with a reasonably regular perimeter which did not exceed 2 mm in diameter. We classified a liver metastasis as a "medium metastasis" if it contained colonies of neoplastic cells that formed a diameter between 2-6 mm and had no evidence of being formed by a fusion of

other metastases. Observed metastases that contained large accumulations of neoplastic cells with irregular shape, a measured diameter exceeding 6 mm, and/or there were indications of possible fusions of adjacent metastases, the metastasis was labeled as a "large metastasis".

Immunohistochemistry

To find non-visible metastases and verify the presence of colon cancer cells, histological analysis of liver and spleen sections were performed at the Center for Environmental Disease's (CRED) Histology Core Facility at the UT/MD Anderson Cancer Center in Smithville, TX with the assistance of Dr. Claudio Conti, DVM. Histological slides of random liver sections from each mouse were stained with hematoxylin and eosin (H&E) and examined to detect non-visible metastases.

Detected liver metastases, labeled "micrometastases", were defined as small nests of tumoral cells not evident by the naked eye or exceeding a number of 12 neoplastic cells that typically form a neoplastic embolus inside blood vessels. Following a positive identification of metastases in the H&E slides, cytokeratin (CK) 20 (sc-17113, Santa Cruz Biotechnology, Santa Cruz, CA), a protein expressed in the human colon but not the mouse liver, was used to confirmed the presence of human colon cancer cell hepatic metastases. For each mouse, two random liver section slides were stained with CK- 20. Two more random slides were selected and stained with CK-20 if the first two appeared negative.

Liver degenerative changes were also determined to assess vitamin A toxicity. These changes consisted of granular degenerative changes, vacuolization, and fatty

deposition. In some cases, areas of frank necrosis and regeneration were observed. Based on the severity of those changes, we define them arbitrarily as (1) mild (small area of the liver is affected with degenerative changes but without the presence of necrotic areas), (2) moderate (degenerative changes covers considerable areas of the liver and small areas of necrosis can be observed), and (3) severe (extensive areas showing degenerative changes with significant areas of necrosis and regeneration). The definition of degenerative changes for each group was objectively labeled based on the mean category of degenerative changes observed in each group.

Statistical Analysis

Values shown are mean ± SEM. Body weight, food intake, and tumor multiplicity were analyzed using two-tailed *t*-tests comparing each vitamin A concentration to parental control (2,400 IU/kg diet) or caPI3K control (2,400 IU/kg diet). Fisher's exact test was used for the statistical analysis of tumor incidence. Significant differences were defined as a *P*-value less than or equal to 0.05.

RESULTS

Food Intake and Body Weight Analysis

The effect of dietary vitamin A supplementation and cell type injected on body weight and feed intake is shown in Fig. 3.1 and Fig. 3.2. At the beginning of the study, each group of mice had a mean body weight of 19.2 ± 0.04g. During weeks 4 and 6 the

mice injected with parental HCT-116 cells and consuming the 100,000 IU vitamin A/kg diet exhibited increased body weight when compared to the mice injected with parental HCT-116 cells consuming the control diet (2,400 IU vitamin A/kg diet (Fig.3.1A). In addition, during the week of the tumor injection, the average body weight of the groups injected with parental HCT-116 cells and supplemented with 12,000 and 100,000 IU vitamin A/kg were higher (20.4 ± 0.21g and 20.1 ± 0.27g, respectively) than the group injected with parental cells consuming the control diet (19.0 ± 0.34g) (Fig.3.1A). At the end of the study, five weeks following tumor injection, the average body weight of the group injected with parental HCT-116 colon cancer cells and consuming the diet containing 100,000 IU vitamin A/kg (20.8 ± 0.48g) diet was greater than the control group (19.5 ± 0.38g) (Fig.3.1A) for mice injected with parental HCT-116 cells. With respect to mice injected with caPI3K-expressing HCT-116 cells, mice consuming 12,000 IU vitamin A/kg diet weighed more (19.1 ± 0.33g) than the group consuming 2,400 IU vitamin A/kg diet (18.2 ± 0.28g) in week 2 of the study (Fig.3.2A). The validity of these results is questionable give that t-tests were used to determine significance. Future analysis using repeated-measures ANOVA will decrease the probability of Type I error, which likely accounts for the significance seen here.

Dietary vitamin A supplementation level did not affect the feed intake of mice injected with parental HCT-116 cells (Fig. 3.1B). In contrast, in week 4, prior to tumor cell injection, the mice consuming the control diet, scheduled for injection with caPI3K-expressing HCT-116 cells had a higher food intake (26.0 ± 0.74g) than mice consuming the 100,000 IU vitamin A/kg supplemented diet scheduled for injection with HCT-116 cells expressing caPI3K (23.7 ± 0.17g) (Fig. 3.2B), as indicated above, this may be due to

a Type I error. However, in the remaining weeks prior to and following week 4, there were no differences in feed intake due to dietary vitamin A supplementation in the mice injected with HCT-116 cells expressing caPI3K (Fig. 3.2B). Because body weight and feed intake did not decrease the amount of vitamin A supplementation, these data show that dietary vitamin A supplementation did not adversely affect body weight and food intake in mice in the current study.

PI3K arbitrates the effects of vitamin A on metastatic incidence

Previously, we have shown that vitamin A supplementation decreases hepatic metastasis incidence in a xenograft mouse model injected with parental HCT-116 cells (manuscript in preparation). In addition, we have shown that PI3K regulates the ability of retinol to decrease HCT-116 colon cancer cell invasion *in vitro* (manuscript in preparation; Fig. 1.3). To determine if PI3K mediates the ability of retinol to reduce metastasis *in vivo,* we assessed the incidence of hepatic metastases in BALB/cAnNCr-nu/nu mice injected with parental PI3K or caPI3K HCT-116 human colon cancer cells. Immunohistochemistry of liver sections stained with H&E were used to identify metastases (Fig. 3.3A and B). Following positive detection of hepatic metastases, CK-20 was used to confirm the presence of human colon cancer cells (Fig. 3.3C and D). As seen in Table 3.2, dietary supplementation with 12,000, 25,000, and 100,000 IU vitamin A/kg diet in mice injected with parental PI3K HCT-116 cells, decreased the incidence of metastasis in a dose responsive manner. The highest reduction in hepatic metastasis was observed in mice injected with parental HCT-116 cells consuming 100,000 IU vitamin

A/kg diet. Specifically, mice consuming 100,000 IU vitamin A/kg diet exhibited a metastatic incidence of 56.8% that of mice consuming the control diet (2,400 IU vitamin A/kg diet) and injected with parental HCT-116 cells (Table 3.2). In contrast, metastatic incidence did not decrease with increasing levels of dietary vitamin A in mice injected with caPI3K HCT-116 cells (Table 3.3). These data show that vitamin A decreases the metastatic incidence in colon cancer cells expressing wild-type PI3K, but not caPI3K. Taken together, this indicates that the ability of vitamin A to decrease the hepatic metastasis of colon cancer is due to the ability of retinol to decrease PI3K activity, confirming our *in vitro* results (Fig 1.3).

Metastatic tumor multiplicity is not affected by dietary vitamin A supplementation or expression of caPI3K

Previously, our laboratory has shown in a chemopreventative study that supplementation with 200,000 IU vitamin A/kg decreased metastatic multiplicity in a nude xenograft model (manuscript in preparation). Therefore, visible liver metastases (white nodules) and micro-metastases were counted in the current study elucidate if PI3K arbitrates the inhibitory effects vitamin A supplementation on metastatic multiplicity *in vivo.* In the current study, tumor multiplicity was defined as the average number of hepatic tumors per mouse for each vitamin A supplemented group (Fig.3.4A and B). We did not observe a dose-responsive decrease in tumor multiplicity as dietary vitamin A content increased in mice injected with either the parental (Fig. 3.4A) or caPI3K-expressing (Fig. 3.4B) HCT-116 cells. These data show that, in the current study,

vitamin A supplementation does not inhibit tumor multiplicity in mice injected with either parental or caPI3K HCT-116 human colon cancer cells.

Tumor size is not affected by vitamin A supplementation or expression of caPI3K.

A chemopreventative agent may affect either the establishment of initial, distant metastases, the growth of these metastases, or both. If an agent decreases the establishment of metastases, fewer small metastases would be seen following treatment of that chemotherapy. To determine where retinol is affecting the metastatic process, we classified each tumor according to size. As can be seen in Table 3.4, we did not observe any significant effects of dietary vitamin A level or cell type on tumor size, thus the point at which vitamin A effects the metastatic process remains unknown.

Assessment of Vitamin A Toxicity

Ingestion of excess levels of vitamin A may result in toxicity as evidenced by dry skin and liver damage. Dry skin occurred in one mouse consuming 25,000 IU vitamin A/kg diet and two mice consuming 100,000 IU vitamin A/kg diet. All of these mice were injected with parental HCT-116 cells, but the cell type should not affect skin condition. Mice consuming 100,000 IU vitamin A/kg diet and injected with caPI3K-expressing cells exhibited a "severe" degree of liver degeneration as diagnosed by a veterinary pathologist. This is potentially due to the high level of vitamin A supplementation combined with the injection of caPI3K cells. All other groups of mice exhibited mild to moderate degrees of degeneration regardless of diet and cell line injected. Importantly,

immunohistochemical analysis for liver cirrhosis did not observe cirrhotic fibrosis in any of the mice in this study.

DISCUSSION

Previous work in our laboratory has shown that retinol decreases the invasion of ATRA-resistant human colon cancer cell via a novel retinoic acid receptor (RAR)-independent mechanism *in vitro* (Park et al. 2007) and that vitamin A supplementation decreases colorectal cancer metastasis in a mouse xenograft model (Park and Lane, in preparation). In the current study, we found that supplementation with 100,000 IU vitamin A/kg diet tends to decrease the incidence of liver metastases in female BALB/cAnNCr-nu/nu nude mice intrasplenically injected with ATRA-resistant HCT-116 cells (Table 3.2) which agrees with metastatic incidence data obtained from an *in vivo* experiment performed in our laboratory (Park and Lane, in preparation) and studies by other groups that have shown elevated vitamin A supplementation reduces metastatic progression of melanoma and pancreatic cancer (Weinzweig et al. 2003; Delage et al. 2004).

Activation of PI3K is associated with an invasive phenotype in human colon cancer (Stephens et al. 2005). Recent literature has shown a relationship supporting a significant role of PI3K in tumor progression *in vivo*. For example, inactivation and silencing of the PI3K inhibitor, PTEN resulted in an increase in tumor metastasis incidence and reduction in survival in a mouse model of thyroid cancer (Guigon et al. 2009). In addition, induced PTEN deficiency showed a significant increase in the

activation and expression of invasion-related proteins downstream of PI3K, such as Akt, mammalian target of rapamycin (mTOR), and nuclear factor-kappaB (NF-κB) (Guigon et al. 2009). Conversely, the PI3K inhibitors, LY294002 or wortmannin, delayed tumor progression and blocked metastasis in a mouse model of thyroid and pancreatic cancer, respectively (Furuya et al. 2007; Teranishi et al. 2009). Our laboratory has shown that retinol inhibits PI3K (Park et al. 2008) and colon cancer cell invasion *in vitro* (Park et al. 2007). The current study expands this work to an in vivo experiment.

In the present study we assessed if PI3K mediates the inhibitory effects of vitamin A supplementation on colon cancer metastasis. PI3K is somatically mutated in over 25% of colorectal tumors and amplification of genomic regions containing PI3K genes has been reported (Samuels et al. 2005). Previous colon cancer nude mouse models have shown that expression of *PIK3CA* mutations in colon cancer cells display enhanced liver metastatic incidence and tumor formation compared to parental PI3K colon cancer cells (Samuels et al. 2005; Guo et al. 2007). Our lab previously shown that PI3K arbitrates the ability of retinol to decrease colon cancer cell invasion *in vitro* (manuscript in preparation, see Fig. 1.3). We did not observe a relatively higher incidence and number of metastases in the caPI3K group versus the parental PI3K cell group in contrast to recent literature. However, it is important to note that different cell preparations were used for the parental and caPI3K groups, therefore direct comparisons between the parental and caPI3K are not possible. Here we demonstrated that mice injected with HCT-116 cells expressing caPI3K did not result in a reduction in hepatic tumor incidence observed in parental HCT-116 group, regardless of vitamin A supplementation as (Table 3.3). This suggests that the ability of vitamin A to decrease metastatic incidence is

arbitrated by PI3K activity. Taken together, these data affirm our previous *in vitro* studies and the role of PI3K in metastasis, and suggest the mechanism by which vitamin A reduces tumor metastasis may involve PI3K and retinol interactions. Future studies are warranted to examine the exact mechanism by which vitamin A inhibits PI3K *in vivo.*

Several studies have reported inhibition of PI3K results in a reduction of tumor formation and progression (Furuya et al. 2007; Teranishi et al. 2009; Wang et al. 2009). Our laboratory has similarly shown that vitamin A supplementation also decreases tumor multiplicity in mice injected with HCT-116 human colon cancer cells (Park and Lane, in preparation). Interestingly, in the current experiment, although we saw a slight decrease in tumor multiplicity in mice injected with parental HCT-116 cells consuming 100,000 IU vitamin A/kg diet, this reduction was not statistically significant (Fig. 3.4). In contrast, in the groups with caPI3K HCT-116 cells there was no distinguishable pattern of the levels of tumor multiplicity when comparing the different dietary vitamin A levels (Fig. 3.4). We can speculate that the difference between the previous and current studies could be attributed to the different cell preparation and/or due to the lower amount of HCT-116 cells injected in the present study.

PI3K plays a key role in the regulation of many cellular processes including proliferation, cell survival, carbohydrate metabolism, and motility (Stein 2001) and therefore may provide an important chemopreventative target to reduce the growth and survival of metastases in cancer. For instance, Rychahou et al. 2006 reported that nude mice intrasplenically injected with HT-29 colon cancer cells and PI3K-specific siRNA treatment resulted in growth inhibition of hepatic tumor metastases (Rychahou et al. 2006). Similarly, inhibition of PI3K via administration of the specific PI3K inhibitor,

LY294002, resulted in a significant reduction in thyroid tumor growth and tumor cell proliferation (Furuya et al. 2007). Retinoids have been shown to inhibit tumor growth in various cancers. For example, intravenous injection of ATRA-incorporated cationic liposome/IL-12 pDNA complexes decreases the number of metastatic tumor cells in a mouse model of metastatic lung tumors (Charoensit et al. 2010). Previously we have shown that retinol inhibits growth of ATRA-resistant HCT-116 human colon cancer cells *in vitro*. In the present study we saw a general pattern of decreased number of medium and large metastases of the 12,000, 25,000, and 100,000 IU vitamin A/kg diet groups injected with parental or caPI3K HCT-116 cells when compared to the respective 2,400 IU vitamin A/kg controls (Table 3.4). This may be due to vitamin A mediated inhibition of tumor growth which results in smaller tumors. However, the apparent changes in tumor size were not statistically significant which is most likely due to variance within groups. Therefore, these data do not definitively show that vitamin A has an effect on the metastatic progression of human colon cancer via decreased tumor growth.

The recommended daily allowance (RDA) levels for vitamin A is 2,331 IU/day for female adults 14 years and older and the upper limit (UL) has been reported around 10,000 IU/day (Food and Nutrition Board 2000). Reports of vitamin A toxicity have resulted in side effects such as fatigue, cachexia, weight loss, dry skin, pruritus, hepatomegaly, splenomegaly, and liver tissue damage (i.e. necrosis and cirrhosis) (Food and Nutrition Board 2000). Chronic vitamin A toxicity can occur from daily ingestion of 25,000 IU or more over a minimum 6 year period to consumption of more than 100,000 IU vitamin A for more than six months (Penniston et al. 2006). However, due to an

excessive amount of variability in the required vitamin A intake values among individuals, it is difficult to establish non-toxic vitamin A levels (Hathcock et al. 1990).

Therefore in the current study we evaluated the body weight, food intake, and liver health status to assess vitamin A toxicity in the animal subjects. In addition, we determined if a lower level of vitamin A supplementation would decrease colon cancer metastasis. We established our control vitamin A diet based on the recommended vitamin A dietary level from the National Research Council for mice (2,400 IU of vitamin A/kg diet) (National Research Council (U.S.). Subcommittee on Laboratory Animal Nutrition. 1995). The increasing levels of vitamin A in the diets used in this study contained 2,400 (1X NRC), 12,000 (5X NRC), 25,000 (10X NRC), and 100,000 (40X NRC) IU vitamin A/kg diet.

If the NRC recommendation for mice is proportional to the RDA for humans, only 2,400 IU vitamin A/kg diet is classified in the safe recommended range. However, our lab has previously shown that supplementation with 200,000 IU vitamin A/kg diet decreased tumor progression in a xenograft model without adversely affecting body weight or food intake, or showing extensive signs of vitamin A toxicity (Park and Lane, in preparation). Other literature has reported ingestion of 150,000 IU (40X NRC) vitamin A/kg diet as retinyl palmitate eliminated malignant melanoma metastasis in mice (Weinzweig, 2003) and 200,000 IU (87X NRC) vitamin A/kg diet decreased aberrant crypt foci incidence in rats (Delage et al. 2004). However, vitamin A toxicity was not reported.

In the current study, we observed higher average body weights in the vitamin A supplemented parental and caPI3K injected groups before the surgeries which were

unexpected. In addition, we found the mice in the parental HCT-116 group consuming the 100,000 IU vitamin A/kg diet exhibited a higher average body weight when compared to the control diet at the end of the experimental study. However, these differences may be due to a Type I error in the analysis, therefore a repeated-measures ANOVA will be conducted in the future to determine if the results are valid statistically. A common symptom in cancer associated with tumor growth is poor appetite and cachexia (A.C.S. 2009). Therefore, the observed difference in average body weight between the 2,400 IU vitamin A/kg diet and the highest dietary vitamin A supplementation (100,000 IU vitamin A/kg) may be a result of the increased tumor metastases exhibited by the mice injected with parental HCT-116 cells and consuming the control diet.

Mice were evaluated for cutaneous signs of vitamin A toxicity throughout the study. Collectively, three of mice in the experiment exhibited dry skin. Liver degenerative changes were also evaluated as signs of vitamin toxicity. The highest degree of liver degeneration was noticed in the caPI3K injected mice consuming the consuming 100,000 IU vitamin A/kg, which exhibited "severe" degenerative changes including significant areas of necrosis and liver regeneration. However, immunohistochemical analysis for liver cirrhosis did not observe cirrhotic fibrosis in any of the mice in either treatment groups. Therefore the liver degeneration observed in the caPI3K groups could be a result of the damaging effects of the metastatic tumors.

In summary, we show that supplementation with vitamin A decreases tumor metastatic incidence in mice injected with parental HCT-116 cells but not HCT-116 cells expressing caPI3K. This suggests PI3K mediates the effects of vitamin A supplementation on liver metastasis incidence *in vivo* which confirms our *in vitro* studies

showing retinol acts upon PI3K to decrease cell invasion. The current experiment elucidates a part of the mechanism by which vitamin A inhibits metastasis, however future research is warranted to further explain the details of this inhibitory pathway.

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Table 3.1. Diet Composition.

Dietary vitamin A (IU/kg)	2,400		12,000		25,000		50,000		100,000		200,000	
	gm%	kcal%	gm%	kcal%	gm%	kcal%	gm%	kcal%	gm%	kcal%	gm%	kcal%
Protein	20	21	20	21	20	21	20	21	20	21	20	21
Carbohydrate	66	68	66	68	66	68	66	68	66	68	66	68
Fat	5	12	5	12	5	12	5	12	5	12	5	12
kcal/gm, Total	3.90	100.00	3.90	100.00	3.90	100.00	3.90	100.00	3.90	100.00	3.90	100.00
Ingredient	gm	kcal	gm	kcal	gm	kcal	gm	kcal	gm	kcal	gm	kcal
Casein	200	800	200	800	200	800	200	800	200	800	200	800
DL-Methionine	3	12	3	12	3	12	3	12	3	12	3	12
Corn Starch	150	600	150	600	150	600	150	600	150	600	150	600
Sucrose	500	2000	500	2000	500	2000	500	2000	500	2000	500	2000
Cellulose, BW200	50	0	50	0	50	0	50	0	50	0	50	0
Corn Oil	50	450	50	450	50	450	50	450	50	450	50	450
Mineral Mix S10001	35	0	35	0	35	0	35	0	35	0	35	0
Vitamin Mix V13001, no added vitamin A	10	40	10	40	10	40	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0	2	0	2	0	2	0
Retinyl Palmitate, 250,000 IU/gm	0.0096	0	0.048	0	0.1	0	0.2	0	0.4	0	0.8	0
TOTAL	1000.059	3902	1000.098	3902	1000.15	3902	1000.25	3902	1000.45	3902	1000.85	3902

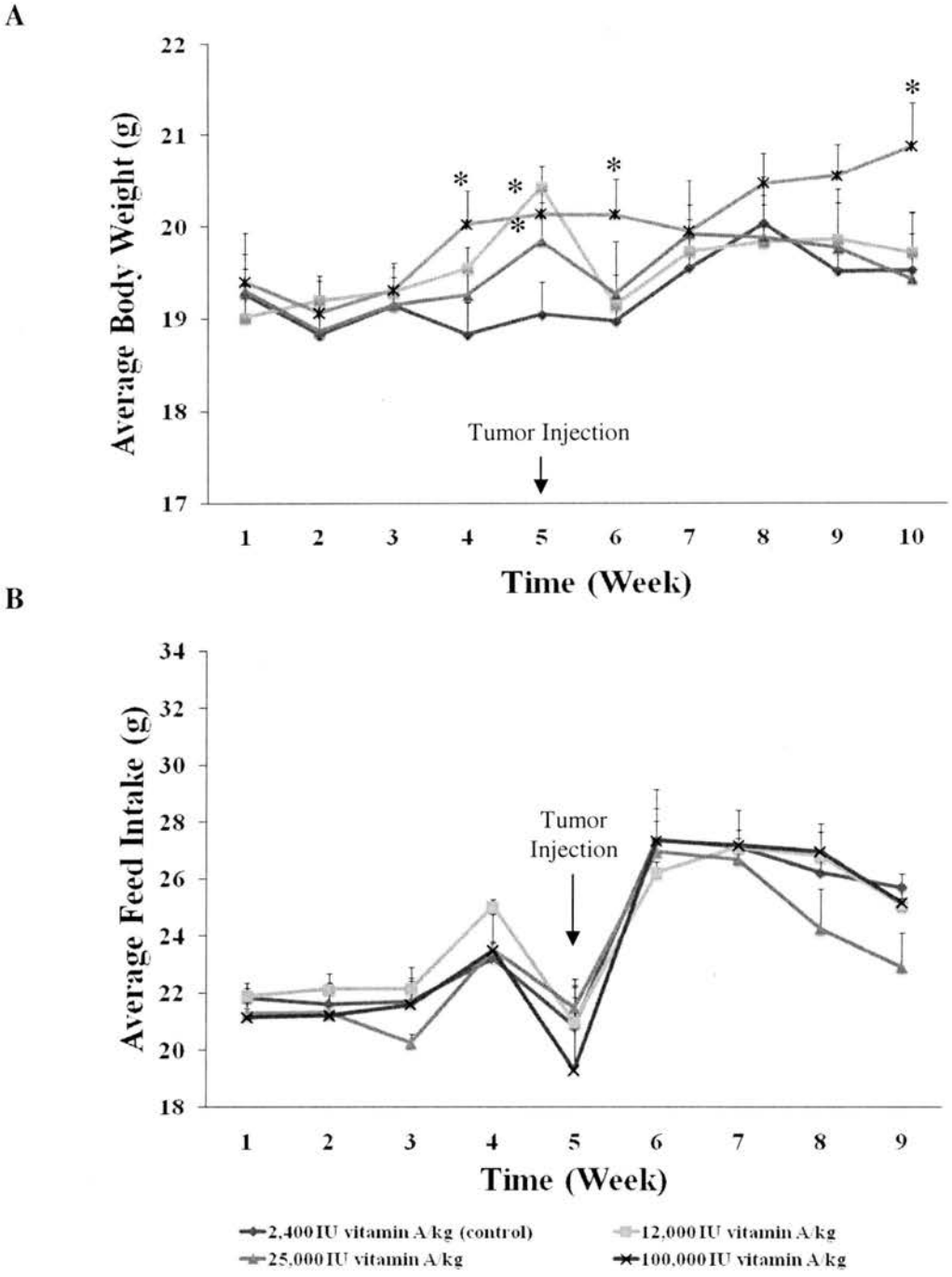
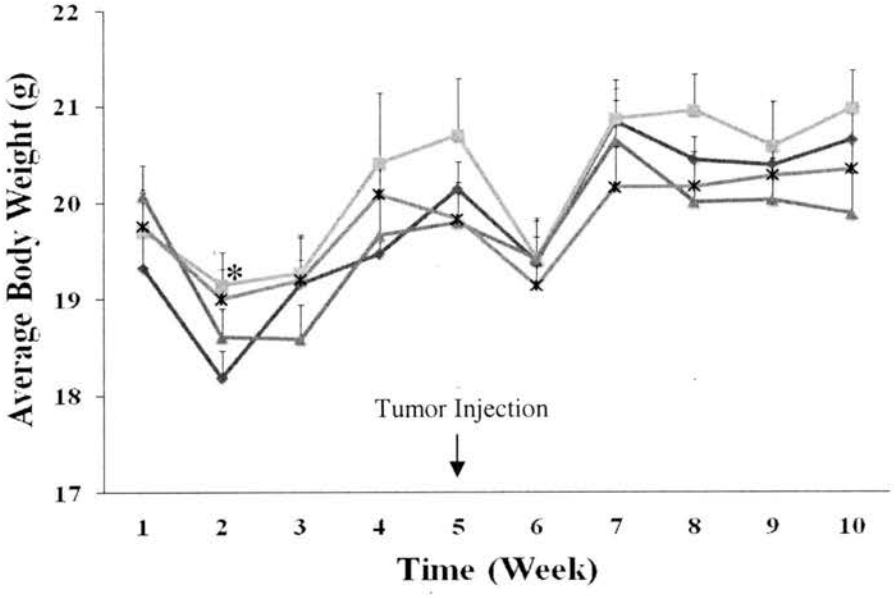


Figure 3.1. Average body weight and food intake of mice injected with parental HCT-116 human colon cancer cells. Average body weight (**A**) and average food intake (**B**) over time for mice injected with parental HCT-116 colon cancer cells consuming vitamin A supplemented diets. Mice were fed 2,400, 12,000, 25,000, 100,000 IU vitamin A/kg diet. Data shown are mean ± SEM for n=10 for the 2,400 IU vitamin A/kg diet group and n=11 for the 12,000, 25,000, and 100,000 IU vitamin A/kg diet groups. *Significantly different from parental 2,400 IU vitamin A/kg diet ($P < 0.05$).

A



B

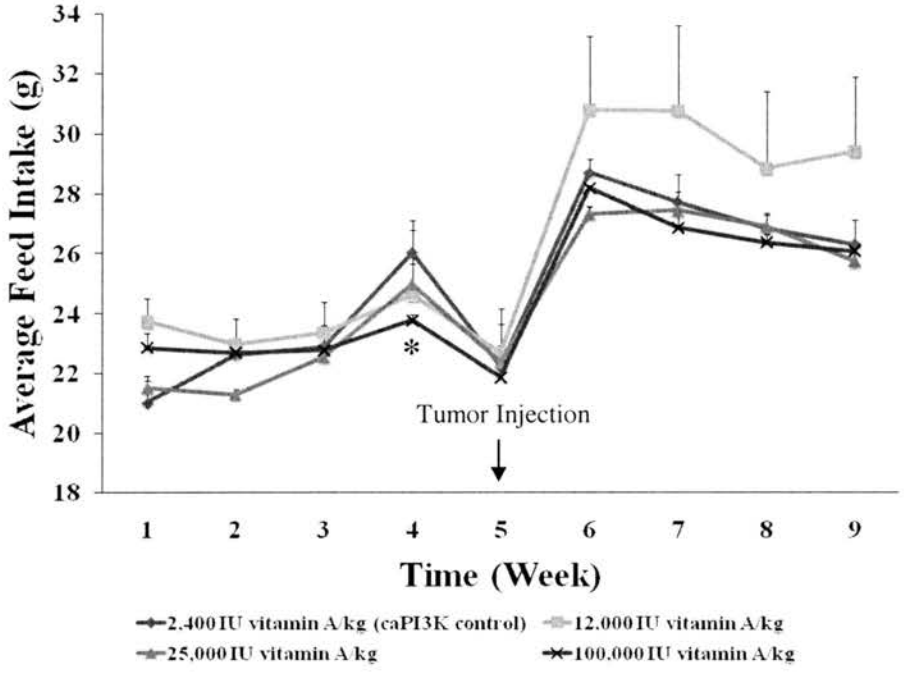


FIGURE 3.2. Average body weight and food intake of mice injected with HCT-116 human colon cancer cells expressing caPI3K. Average body weight (**A**) and average food intake (**B**) over time for mice injected with HCT-116 colon cancer cells expressing caPI3K consuming vitamin A supplemented diets. Mice were fed 2,400, 12,000, 25,000, and 100,000 IU vitamin A/kg diet. Data shown are mean ± SEM for n=11 for the 2,400 and 25,000 IU vitamin A/kg diets and n=12 for the 12,000 and 100,000 IU vitamin A/kg diet groups. *Significantly different from caPI3K 2,400 IU vitamin A/kg diet ($P < 0.05$).

Table 3.2. Dietary vitamin A content versus incidence of metastases in mice injected with parental HCT-116 cells.

Dietary Vitamin A (IU/kg diet)	Number of mice with metastases per group	Tumor Incidence (% parental control)
2,400 (control)	8/10	100
12,000	6/11	68.1
25,000	7/11	79.5
100,000	5/11	56.8

Table 3.3. Dietary vitamin A content versus incidence of metastases in mice injected with HCT-116 cells expressing caPI3K.

Dietary Vitamin A (IU/kg diet)	Number of mice with metastases per group	Tumor Incidence (% caPI3K control)
2,400 (control)	5/11	100
12,000	8/12	146.6
25,000	7/11	140
100,000	6/12	110

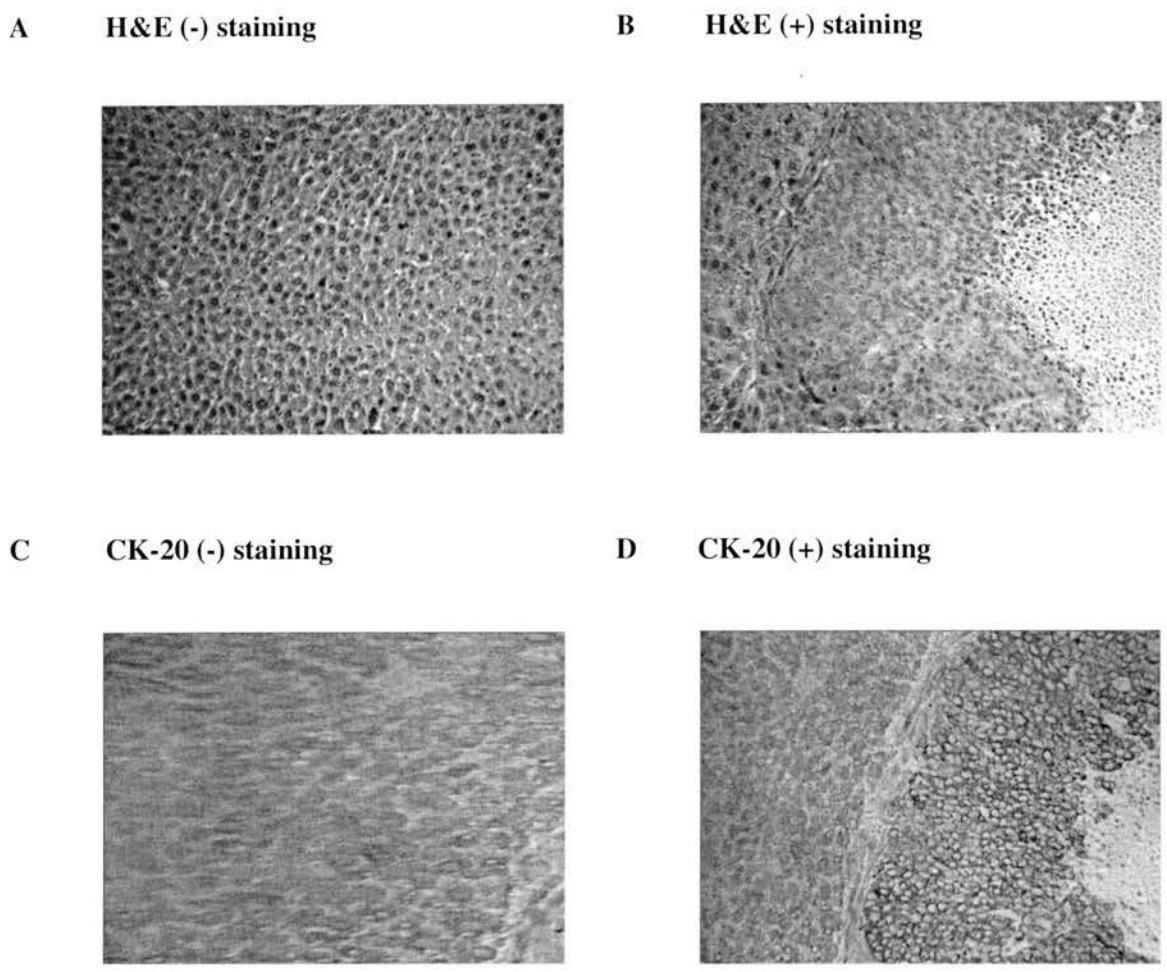
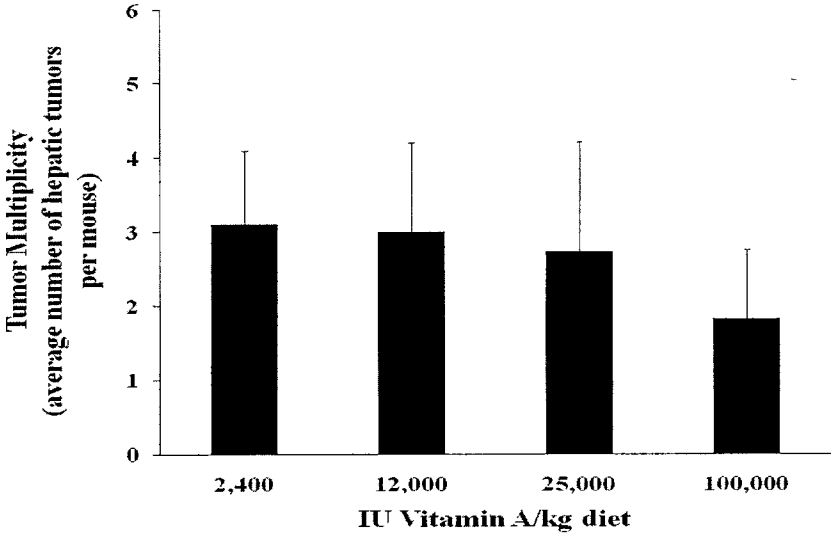


Figure 3.3. Representative Immunohistochemical data. Consecutive slides of liver metastatic tumors were stained with H&E (**A, B**) and CK-20 (**C, D**). Negative (normal liver) (**A,C**) and positive (liver metastasis) (**B, D**). One representative sample is shown.

A



B

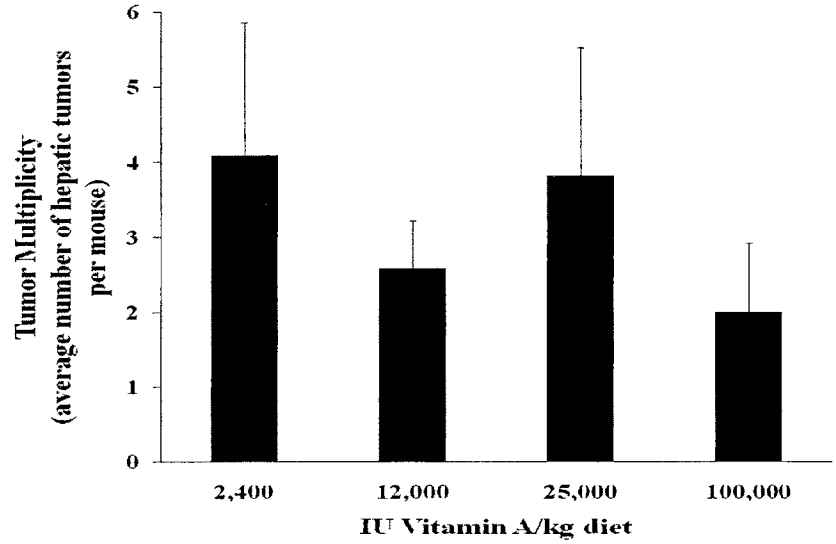


Figure 3.4. Dietary vitamin A supplementation does not reduce tumor multiplicity. BALB/cAnNCr-nu/nu female mice, aged 6 to 8 weeks, were randomly divided into four vitamin A diet groups. The control group consumed a diet containing 2,400 IU vitamin A/kg. The other three groups consumed diets supplemented with 12,000, 25,000, and 100,000 IU vitamin A/kg as retinyl palmitate. These diets were consumed *ad libitum* for one month prior to tumor generation via intrasplenic injection with 1 x 10^6 parental or caPI3K HCT-116 cells. Diets consumption continued until sacrifice. Average tumor multiplicity per mouse in the parental (**A**) and caPI3K (**B**) HCT-116 cell injected groups was expressed as mean ± SEM for n=10 for the 2,400 IU vitamin A/kg diet and n=11 for the 12,000, 25,000, and 100,000 IU vitamin A/kg diet groups injected with parental HCT-116 cells; for n=11 for the 2,400 and 25,000 IU vitamin A/kg diets and n=12 for the 12,000 and 100,000 IU vitamin A/kg diet groups injected with caPI3K HCT-116 cells.

Table 3.4. Observed changes in hepatic tumor size of mice injected with parental or caPI3K HCT-116 cells.

	Average Number of Metastases (per mouse)							
Cell injection	Parental HCT-116 cells				caPI3K HCT-116 cells			
Tumor Size	2400 IU vitamin A/kg diet	12000 IU vitamin A/kg diet	25000 IU vitamin A kg diet	100000 IU vitamin A/kg diet	2400 IU vitamin A/kg diet	12000 IU vitamin A/kg diet	25000 IU vitamin A kg diet	100000 IU vitamin A kg diet
Micro	0.6 ± 0.27	0.18 ± 0.18	0.18 ± 0.18	0.27 ± 0.19	0.82 ± 0.35	0.42 ±0.23	0.54 ± 0.31	0.33 ± 0.19
Small (< 2 mm)	1 ± 0.47	1.82 ±0.88	1.54 ± 1.25	0.64 ± 0.31	2.27 ± 1.07	1.58 ± 0.47	1.64 ± 0.83	1 ± 0.72
Medium (2-6 mm)	0.8 ± 0.33	0.54 ± 0.31	0.73 ± 0.36	0.45 ± 0.28	0.82 ± 0.5	0.5 ± 0.34	1.18 ±0.39	0.58 ± 0.29
Large (> 6 mm)	0.7 ± 0.3	0.45 ± 0.28	0.27 ± 0.19	0.45± 0.25	0.18 ± 0.18	0.08 ± .08	0.36 ± 0.20	0.08 ± 0.083

IV. DISCUSSION AND FUTURE DIRECTIONS

Elevated tumor invasion and metastasis is associated with reduced disease free survival (Yu et al. 1997). Therefore, an increased amount of attention has been placed on investigating therapies that prevent or reduce the metastatic progression of colorectal cancer. Numerous adjuvant chemotherapies have been developed, however most all lead to several unwanted side effects (e.g. nausea, diarrhea, and poor appetite) (A.C.S. 2009). For these reasons, a more purposeful and efficient treatment for colon cancer is warranted. Numerous studies have investigated the ability of bioactive dietary components to prevent and treat colon cancer without producing severe detrimental side effects. Here we evaluated the ability of dietary vitamin A to prevent metastasis and slow the progression of colorectal cancer. Chapter 1 introduced the process of metastasis and the invasion-related proteins that influence cancer progression. It also described the ability of retinoids to inhibit cell invasion and metastasis, and explained why we chose to study retinol. Finally, the introduction provided information concerning the effects of retinol on specific proteins involved in cancer cell invasion and provides a hypothetical model showing the mechanism by which retinol inhibits metastasis of colon cancer.

Chapter 2 showed that PI3K activation mediates the ability of retinol to inhibit ATRA-resistant HCT-116 human colon cancer cell invasion and invasion-associated proteins. Specifically, caPI3K and caAkt mediated the ability of retinol to decrease

MMP-9 activity and protein, and increase TIMP-1 protein levels. In addition, caAkt, but not caPI3K, was able to regulate the ability of retinol to reduce β-catenin. However, the ability of retinol to decrease human colon cancer cell invasion was independent of β-catenin. Finally, expression of caPI3K was unable to block the inhibitory effects of retinol on Akt activity. Similar results have been observed in other caPI3K models that showed decreases in levels of phosphorylated Akt as seen in the current study and suggest PI3K may promote cancer through Akt-dependent and Akt-independent mechanisms (Guo et al. 2007; Vasudevan et al. 2009). These results suggest that because PI3K activity does not result in inhibition of Akt, following treatment with retinol, PI3K and Akt may individually be regulating MMP-9 expression.

The mechanism by which retinol inhibits PI3K activity is not clear. Comparison of the electrostatic potential surfaces of retinol revealed that retinol had a similar electrostatic potential surface profile to the PI3K inhibitor, wortmannin (Park et al. 2008). Previous work has shown that wortmannin interacts with ATP binding site of the p110α catalytic subunit of PI3K to inhibit activity (Wymann et al. 1996). Therefore, it may be likely that retinol may prevent PI3K activation by binding to the ATP binding site of PI3K. Alternatively, retinol may behave similar to Phosphatase and tensin homolog (PTEN) to reduce PI3K activity and its downstream proteins. (PTEN) is a protein phosphatase that acts as a tumor suppressor due to its ability to oppose the actions of PI3K via dephosphorylation [For review, see: (Chalhoub et al. 2009)]. PTEN has been shown to be mutated in several cancers and attributed to elevated PI3K activity (Ali et al. 1999). Previous studies have shown that application

of retinoids increases PTEN expression and activity. For example, ATRA, in combination with interferon-gamma (IFN-γ), increased PTEN expression in LN18 malignant glioma cells (Zhang et al. 2008). In addition, et al. reported that combination of the retinoid, N-(4-hydroxyphenyl) retinamide (4-HPR), and genistein mediated reactivation of PTEN for early cell cycle exit due to G1/S phase arrest in neuroblastoma cells (Janardhanan et al. 2009). More data is needed to determine the precise mechanism by which retinol inhibits PI3K activity.

The ability of retinol to decrease cell invasion was not regulated by β-catenin, however it was found that Akt does mediate the retinol-induced decrease in β-catenin protein levels. Although it appears β-catenin is not involved in the pathway by which retinol inhibits metastasis, the previous work in our laboratory indicates that retinol may reduce tumor growth and proliferation via inhibition of β-catenin gene transcription, specifically of c-myc and cyclin-D_1 (Dillard et al. 2007). We have also shown that retinol inhibits the proliferation of ATRA-resistant human colon cancer cells by slowing the progression from the $G_{0/1}$ to the S phase of the cell cycle (Park et al. 2005), a process mediated by cyclin D_1 (Shah et al. 2002). Similarly, Yu et al. (2007) showed that application of ATRA up-regulated the apoptosis related membrane protein, protein 3, which resulted in the cell cycle arrest at the G1/S phase by decreasing the β-catenin mediated expression of cyclin D_1 (Yu et al. 2007). In addition, supplementation with retinyl palmitate prevented the increase in colonocyte β-catenin due to consumption of a high fat diet (Delage et al. 2005). Overall, the current literature and the data reported herein suggest that although retinol decreases β-catenin levels via inhibition of Akt activity, β-catenin does not appear to play a role

in the colon cancer cell invasion pathway manipulated by retinol. However, retinol may have an inhibitory effect on colon cancer cell growth and proliferation through increased RXR-mediated β-catenin proteasomal degradation and therefore decreased β-catenin mediated transcription of genes such as cyclin D_1 and c-myc. Future research is needed to elucidate the possible role of retinol in reduction of tumor growth and proliferation through inhibition of Akt activity and β-catenin protein levels.

In Chapter 2, we also speculated alternative PI3K and Akt-mediated pathways by which retinol may be modulating MMP-9 levels and hence, cell invasion. Elevated levels of MMP-9 promote metastatic progression in colorectal cancer via intravasation and extravasation (Bernhard et al. 1994; Legrand et al. 1999; Bergers et al. 2000; McCawley et al. 2001; Huang et al. 2002). Retinoids have been shown to reduce invasion and metastasis by decreasing MMP protein levels or activity via inactivation of PI3K and Akt (Liu et al. 2003; Andela et al. 2004; Chung et al. 2004; Cheng et al. 2006; Arcaro et al. 2007; Papi et al. 2007; Kang et al. 2008; Chen et al. 2009) which supports our findings of the inhibitory role of retinol on colon cancer cell invasion via decreased MMP-9.

The link between PI3K and Akt activation and MMP-9 activity is unclear. However, PI3K and Akt pathways may regulate MMP-9 expression through NF-κB activation. In many types of cancer, NF-κB is constitutively active, nonetheless previous literature has reported that inhibition of NF-κB activity reduces MMP-9 activity and expression and metastasis in several cancers, including colorectal cancer

(Huang et al. 2001; Shih et al. 2007; Park et al. 2009; Lu et al. 2010; Yang et al. 2010).

We hypothesize that PI3K may contribute to the activation of NF-κB-mediated MMP-9 expression through a PI3K/PDK-1/PKC/IKK-dependent pathway (Fig. 2.7). PI3K-mediated PDK-1 activity is known to be involved in the phosphorylation and activation of several isoforms of PKC including PKC-delta and PKC-zeta. In particular, PKC-zeta or PKC-delta have been shown to induce NF-κB gene transcription by phosphorylating the IKKβ subunit of the IKK complex and thereby activating NFκB mediated transcription of MMP-9 (Hirai et al. 2003; Hla et al. 2008). Alternatively, PKC activity has been shown to stimulate extracellular signal-regulated kinases (ERK) signaling and consequential activation of activator protein (AP-1) and NF-κB-dependent MMP-9 expression (Hwang et al. 2010). Several studies have supported the roles of PKC-delta and PKC-zeta signaling in the downstream regulation of genes involved in cell proliferation, migration, and invasion in many cancers (Cerda et al. 2006; Lai et al. 2010; Mertens-Walker et al. 2010). Therefore, we believe PI3K activates PDK-1, which in turn phosphorylates IKKβ and releases NF-κB. This cascade allows NF-κB to migrate to the nucleus and initiate gene transcription of MMP-9 (Fig. 2.7). Inhibition of PI3K by retinol would inhibit this pathway, thereby decrease metastasis.

In addition, Akt may also play a role in MMP-9 expression through NF-κB. Activation of Akt leads to a plethora of downstream signaling involved in several processes. The Akt/ IKKα signaling pathway results in increased NF-κB mediated gene transcription. Akt has recently been shown to stimulate NF-κB activity

indirectly through an IKKα/β-catenin mediated process in colon cancer cells (Agarwal et al. 2005). Therefore this signaling cascade may be a key contributor to colon cancer progression. We hypothesize that Akt activates NF-κB activity by phosphorylating the IKKα subunit of the IKK complex, which in turn allows IKK to phosphorylate and activate the p65 subunit of NF-κB and induce MMP-9 expression (Fig. 2.7).

TIMP-1 has been shown to reduce cell invasion by inhibiting MMP-9 activity (Weinzweig et al. 2003). We have shown that PI3K and Akt activity mediate TIMP-1 protein levels. The exact link between PI3K/Akt and TIMP-1 expression is unclear. The signaling growth factor, TGF-β1 has been shown to mediate TIMP-1 expression in normal and cancerous tissues (Sehgal et al. 1999; Hall et al. 2003; Kwak et al. 2006; Offenberg et al. 2008). Recently, Bian et al. 2009 reported that TGF-β1 signaling and PI3K/Akt pathway cross-talk in mice with head and neck carcinogenesis (Bian et al. 2009) which implicates a possible link between PI3K/Akt and TIMP-1 expression. Therefore we believe PI3K/Akt signaling may contribute to the expression of TIMP-1 protein, possibly mediated by TGF-β1.

Ultimately, we hypothesize PI3K and Akt independent signaling pathways activate NF-κB transcriptional activity, and consequently result in a increase in MMP-9 expression and decrease in TIMP-1 protein levels (Fig. 2.7). In concurrence with recent literature and our findings, we believe retinol blocks this pathway and its downstream proteins to subsequently inhibit colon cancer cell invasion.

Future studies are warranted to examine this hypothetical model (Fig. 2.7) and determine if the mechanism by which retinol reduces colon cancer metastasis

involves direct or indirect interaction with these downstream invasion-related proteins. To examine this pathway, we could measure the levels of MMP-9 activity and protein and simultaneously the levels of the intermediate proteins hypothesized to regulate MMP-9 expression following treatment with retinol. If treatment with retinol results in a decrease in MMP-9 expression and levels of that particular protein, we can speculate it is involved in the metastatic pathway inhibited by retinol. From there, we could use constitutively active, dominant negative, or knock out models to determine specifically how the actions of retinol modulate that protein and the entire pathway.

In Chapter 3, our observations concerning the role of PI3K in cell invasion and the ability of retinol to decrease PI3K activity in the previous chapter were confirmed *in vivo.* We showed that the ability of retinol to inhibit PI3K mediates the ability of dietary vitamin A supplementation to decrease hepatic tumor incidence in a chemopreventive xenograft mouse model. In Chapter 2, we found that expression of caPI3K did not block the inhibitory effect of retinol on Akt phosphorylation *in vitro* which suggests retinol may be directly inhibiting Akt as well as PI3K. Similar results have been observed in other caPI3K models. For instance, mutant *PI3CA*-bearing colon cancer cells treated with a PI3K inhibitor showed decreases in levels of phosphorylated Akt as seen in the current study (Guo et al. 2007). In addition, Vasudevan et al. 2009 suggests, after evaluating phosphor-protein profiling and genomic studies in *PIK3CA*-mutant cancer cell lines, PI3K may promote cancer through Akt-dependent and Akt-independent mechanisms (Vasudevan et al. 2009). These data support the theory that PI3K may promote cancer through Akt-dependent

and Akt-independent mechanisms. Previous studies indicate that Akt over-expression/activation is highly correlated to human colorectal cancer (Johnson et al. 2010) and inactivation of Akt markedly reduced proliferation in HCT-116 human colon cancer cells, thus illuminating the role of Akt in tumor growth (Ericson et al. 2010). Since Akt regulates the ability of retinol to inhibit cell invasion and β-catenin protein levels, and Akt plays an important role in tumor growth, future *in vivo* experiments should investigate the effects of vitamin A on tumor growth and the potential role of Akt in the progression of colon cancer metastasis.

In conclusion, the first part of my work shows that retinol individually inhibits the activities of PI3K and Akt to reduce MMP-9 expression and subsequent cell invasion *in vitro*. The precise metastatic pathway(s) mediated by the actions of retinol in colon cancer remains unknown. Therefore, we also introduced a new hypothetical pathway (Fig. 2.7) that may give insight to the mechanism by which retinol inhibits colon cancer metastasis. The second part of my work expands the *in vitro* work and demonstrates that PI3K mediates the ability of dietary vitamin A to decrease metastasis in a xenograft mouse model. To our knowledge, these studies are the first to elucidate part of the mechanism by which retinol inhibits colon cancer metastatic progression *in vitro*. In addition, we are the first to show the interaction between vitamin A and PI3K in an *in vivo* model of colon cancer metastasis. In summary, my work suggests that dietary vitamin A supplementation prevents colon cancer progression via a non-genomic pathway targeting PI3K, Akt, MMP-9, and TIMP-1. Future studies are required to investigate the proteins between PI3K and Akt

activation and MMP-9 expression and further reveal the specific inhibitory actions of retinol on human colon cancer invasion.

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