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**THE EFFECTS OF GENETIC AND LUMINAL RISK FACTORS ON  
CYCLOOXYGENASE-2 EXPRESSION IN HUMAN COLON CANCER CELLS**

**by**

**Michele Taylor Parker**

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**A Dissertation Submitted to the Faculty of the  
GRADUATE INTERDISCIPLINARY PROGRAM IN CANCER BIOLOGY**

**In partial fulfillment of the Requirements  
For the Degree of**

**DOCTOR OF PHILOSOPHY**

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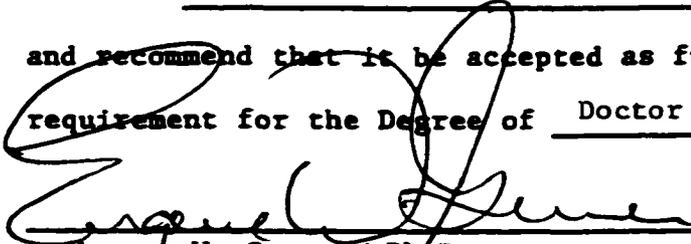
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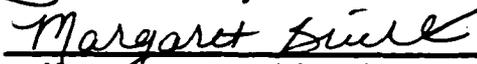
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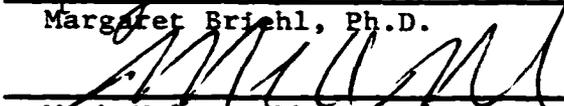
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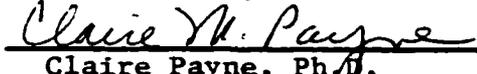
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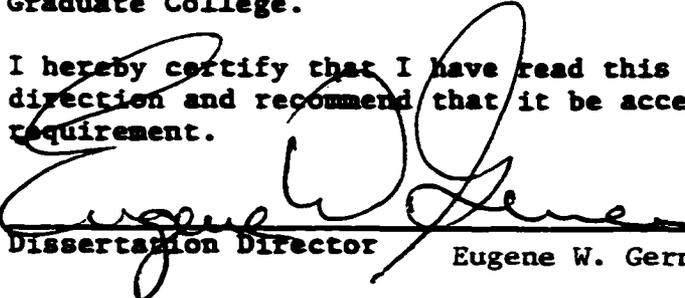
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## **ABSTRACT**

The process of colon carcinogenesis is dependent upon a variety of genetic mutations and intestinal luminal risk factors. These risk factors cooperate to influence pathophysiological targets directly linked to the carcinogenic process. Cyclooxygenase-2 (COX-2) is one such target, and it is found to be upregulated in 50% of colonic adenomas and 85% of carcinomas. The interaction between genetic mutations and luminal risk factors to affect COX-2 expression was explored in this dissertation work.

An activated K-ras oncogene and mutant APC tumor suppressor gene both regulate COX-2 posttranscriptionally. Using colon cancer cells with these genetic alterations, we determined that an activated K-ras stabilizes COX-2 mRNA and works through a PKC-dependent mechanism in a cell line-specific manner. K-ras also affected PTEN expression to potentially suppress cell survival. Wild-type APC downregulated COX-2 protein levels without affecting mRNA. APC interfered with PI-3K-dependent signaling of COX-2, and affected PKC-dependence signaling to further modulate COX-2 expression.

In addition to genetic alterations, luminal factors obtained from the diet influenced COX-2 expression in these colon cancer cell models. Secondary bile acids like deoxycholic acid (DCA) mediated COX-2 protein stability in addition to its known effects on transcription. DCA's effects on COX-2 protein were partially mediated by APC mutational status and PKC-signaling. Polyamines, another luminal risk factor for colon cancer, regulated COX-2 through the induction of RNA processing by the

polyamine-dependent gene, eIF-5A and through a reliance on transport through the cell membrane.

The chemopreventive agent, Sulindac, has two metabolites - sulindac sulfone and sulindac sulfide. Previous to this study, little was known about the mechanism of action of the sulfone metabolite. We determined that sulindac sulfone suppressed COX-2 protein levels, thus impinging on this protein's ability to signal downstream genes or to produce prostaglandins. In addition, DFMO, a chemopreventive agent that suppresses polyamine synthesis, significantly induced COX-2 protein levels, predicating the importance of combination chemoprevention to battle COX-2-driven tumorigenesis. The intricate cooperation of genetic mutations and luminal risk factors affecting COX-2 expression and subsequently tumorigenesis, suggest that multiagent interventions may be necessary for successful strategies of colon cancer chemoprevention.

## **CHAPTER 1: INTRODUCTION AND BACKGROUND**

### **The Process of Colon Carcinogenesis**

Colon cancer is the third most common and second most lethal cancer in both men and women in western societies. The onset of sporadic colon cancers is slow and it may take upwards of 30 years to develop. Risk increases with age and the mean age at presentation is 60 years. These cancers are often discovered at an advanced stage due to lack of availability and use of preventive measures.

Colon carcinogenesis is a multi-step process involving mutations or loss of function in oncogenes and tumor suppressor genes. Multiple, perhaps even hundreds of genetic mutations are required to produce a colon carcinoma, allowing for the long gestation period from a preneoplastic lesion to invasive carcinoma (Fearon and Vogelstein, 1990). The earliest identified precursors to colon carcinoma is the aberrant crypt foci (ACF) which are comprised of actively growing colonic crypts (Pretlow et al., 1991). ACF lesions contain one or more colonic crypts that are larger in size than their normal counterparts and have a thicker epithelial layer (McLellan and Bird, 1988). The normal epithelial lining of the colon is made up of U-shaped crypt structures which have stem cells originating at the bottom of the U. These stem cells slowly migrate up the sides of the crypt and begin differentiating in the middle section. This is usually where initiated

cells will stop and develop into an ACF. Normal cells progress towards the top of the crypt and are sloughed off by apoptosis.

With the occurrence of additional genetic or epigenetic mutations, an ACF can then progress to a benign adenoma, or polyp. There are both villous and tubular polyps. Tubular polyps look as their name suggests, glandular and tube-like; meanwhile, villous tumors appear as finger-like protrusions and are less differentiated, more dysplastic and more likely to progress to an invasive carcinoma. Polyps that are greater in size than 1 cm also have increased likelihood of becoming a cancer. Removal of these polyps decreases risk of carcinoma development (Vamosi-Nagy and Koves, 1993), thus supporting Fearon and Vogelstein's model of colon cancer progression.

An important molecular hallmark of colon cancer is genetic instability. It has been postulated that endogenous mutation rates would not suffice to create the several "hits" in tumor suppressor genes and oncogenes seen in colon carcinogenesis. Thus, some sort of inherent genetic instability must be present in order to accelerate the mutation rate. Sporadic cancers usually induced by mutations in the adenomatous polyposis coli (APC) gene give rise to chromosomal instability (CIN), while hereditary cancers like HNPCC give rise to microsatellite instability (MIN). CIN tumors have a defect in chromosomal segregation which results in differences in chromosome numbers (Lengauer et al., 1997). The integral role of APC in this process will be discussed in the next section. MIN tumors have nucleotide mutation rates that are 2-3 fold higher than in normal cells (Parsons et al., 1993; Bhattacharyya et al., 1994). This is the key to the "mutator phenotype." The "mutator phenotype" is described as an increase in mutation frequency

that underscores the process of carcinogenesis, even in the absence of selective advantage. The nucleotide instability associated with the mutator phenotype occurs in repetitive sequences called microsatellites (Ionov et al., 1993; Thibodeau et al., 1993). A prime example of MIN is found in the genetic colon cancer, HNPCC.

There are two main hereditary colon cancers: hereditary nonpolyposis colon cancer (HNPCC) and familial adenomatous polyposis (FAP). HNPCC accounts for approximately 5% of colon cancers. These tumors generally have a wild-type APC and normal Wnt signaling pathway. The defect, instead, is in the repair of mismatched DNA that occurred in replication. Thus, it is also referred to as “replication error repair” or RER. Two proteins, hMSH2 and GTBP/160, recognize the mismatch in the DNA, recruit additional proteins to this site, including hPMS2 and MLH1, and then incision, excision, resynthesis and ligation ensues. When a mismatch cannot be repaired, the cell continues to divide with its mutated DNA and the organism is more susceptible to cancer. This study used two human colon cancer cell lines derived from HNPCC tumors, HCT-116 and HCA-7 cells. Both cell lines have a wild-type APC and both are considered to be RER+, with MIN (Aaltonen et al., 1994; Abdel-Rahman et al., 2001).

FAP is an autosomal dominant disease and these patients present with hundreds to thousands of polyps by age 20-30. By age 30, one or two of these usually progress to a malignant carcinoma and the patient opts for a complete colostomy to prevent mortality. FAP accounts for 1% of colon cancers in the United States (Petersen et al., 1991). FAP results from the aberrant expression or mutations in the APC tumor suppressor gene.

### **Adenomatous Polyposis Coli (APC)**

APC, or its signaling pathway, is mutated in almost 90% of sporadic colon cancers and 30% of melanoma skin cancers (Barker et al., 2000). The mutation of the APC gene is an early event in colon carcinogenesis and is therefore, considered to be the initiating event. In a normal cell, Wnt growth signals are relayed through the cell membrane via the frizzled receptor and low-density lipoprotein-receptor-related protein LRP6 (Tamai et al., 2000). The signal then inactivates glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) with the help of the dishevelled protein. This inactivation causes the dissociation of the APC/ $\beta$ -catenin/GSK-3 $\beta$  protein complex (Rubinfeld et al., 1996). The dissociation allows for  $\beta$ -catenin to be targeted for degradation by the 26S proteasome, which serves to help keep cell proliferation in check.

However, if APC is mutated, the APC/ $\beta$ -catenin/GSK-3 $\beta$  complex cannot form, causing  $\beta$ -catenin to build up in the cytoplasm and translocate into the nucleus where it can positively influence transcription of growth-promoting genes.  $\beta$ -catenin binds to the Tcf-Lef transcription factor of downstream genes to either promote proliferation or block apoptosis. One such downstream gene of the APC/ $\beta$ -catenin pathway is the *c-myc* oncogene. (He et al., 1998). In many colon cancers, the APC gene is not necessarily mutated but the mutation in the pathway is found in  $\beta$ -catenin, which yields the same constitutive signaling through the pathway.

APC has other roles in the cell in addition to its modulation of the Wnt signaling pathway. APC plays a role in the G<sub>1</sub>/S transition of the cell cycle by interacting with cyclin-dependent kinases (Trzepacz et al., 1997). APC is also important in maintaining

intestinal cell migration up the crypt and modulating apoptosis (Morin et al., 1996; Zhang et al., 2001). In fact, it has recently been shown that mutations in APC induce stem cell overproduction at the base of the crypt, beginning the initiation of ACF formation, the initiating step in colon carcinogenesis (Boman et al., 2001).

APC also plays a role in cell-cell adhesion since it controls intracellular amounts of  $\beta$ -catenin.  $\beta$ -catenin normally complexes with E-cadherin and  $\alpha$ -catenin, two actin-binding proteins, as well as other cell adhesion proteins like plakoglobin (Su et al., 1993; Rubinfeld et al., 1995). By regulating how much  $\beta$ -catenin is able to complex with these proteins, APC is in effect controlling contact between cells. APC also interacts with the microtubule cytoskeleton directly (Mimori-Kiyosue et al., 2000). APC expression is found at the growing ends of microtubules, implicating it in the process of mitosis. Cells expressing mutant APC have an abundance of spindle microtubules that do not connect to the kinetochores and thus results in the CIN phenotype (Fodde et al., 2001). APC plays two roles in mitosis: the proper attachment of the mitotic spindle to the dividing chromosomes at the kinetochore and the regulation of centrosome duplication. It is not surprising, then, that APC mutations would lead to aberrant chromosome numbers and thus genetic instability (Thiagalingam et al., 2001).

### **Signaling Pathways Associated with the Ras Oncogene**

While APC is considered the initiating mutation in colon cancer, K-ras mutations are the genetic event defining the promotion stage. K-ras mutations occur in 18-56% of human colonic adenomas (Otori et al., 1997; Martinez et al., 1999) and 47% of carcinomas (Vogelstein et al., 1988). K-ras mutations occur in about half of colonic

polyps produced by chemical carcinogens in rats (Erdman et al., 1997). Mutations in the p53 tumor suppressor gene, which are associated with the invasive phenotype of colonic neoplasia, are induced at only low levels by carcinogen treatment in rodents (Erdman et al., 1997).

The K-ras oncogene is a member of the membrane associated, guanine nucleotide-binding proteins. The activity of ras depends on its being bound to GTP. Guanine nucleotide exchange factors (GEFs) promote the GTP-bound ras while GTPase activating proteins (GAPs) promote GTP hydrolysis and result in the GDP-bound inactive form of ras (Bourne et al., 1991). The ras family of oncogenes consists of 3 genes, H-ras, K-ras, and N-ras, which are almost identical in sequence homology. These genes differ in their expression patterns in different tissues. All have been found to have point mutations in human cancers including liver, colon, skin, pancreatic, and lung cancers. These point mutations lead to constitutive signaling of genes involved in proliferation, cell survival and remodeling of the actin cytoskeleton.

The activated, GTP-bound form of ras signals a variety of mitogen-induced and stress-induced pathways, leading to transcription of genes involved in cell growth and proliferation (Vojtek and Der, 1998). Mitogens such as growth factors can activate ras through the epidermal growth factor receptor, and stress factors affecting ras include ultraviolet light, heat, and genotoxins. The most prominent of these pathways is the one in which ras binds to the serine-threonine kinase, raf, at the plasma membrane and initiates a cascade of mitogen-induced protein kinase (MAPKs) activities which

culminate in the nucleus with the activation of genes containing Elk-1 transcription factor binding sites.

Raf can also directly activate protein kinase C (PKC) which signals another set of stress-activated kinases that can phosphorylate the c-jun transcription factor. There are 11 identified PKC isoforms, classified into three categories, the classical PKCs ( $\alpha$ ,  $\beta$ I,  $\beta$ II,  $\gamma$ ), the novel PKCs ( $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$ ,  $\mu$ ) and the atypical PKCs ( $\lambda$ ,  $\zeta$ ). They are grouped by which co-factors they need to initiate the phosphorylation cascade. The classical PKCs require both calcium and diacylglycerol (DAG), the novel PKCs require only DAG, and the atypical PKCs are independent of both calcium and DAG. PKC is instrumental in colonic cell growth and differentiation, tumor promotion and apoptosis (Blobe et al., 1994).

Another ras effector gene is phosphoinositol 3-kinase (PI-3K) which initiates a signaling pathway for cell survival (Rodriguez-Viciano et al., 1994). The enzymatic role of PI-3K is to phosphorylate phosphoinositides (PtdIns) at the 3-position of the inositol ring, generating PtdIns3P, PtdIns(3,4)P<sub>2</sub> and PtdIns (3,4,5,)P<sub>3</sub>, which can lead to further activation of second messengers like cAMP, aiding in the transmission of signals for proliferation to the nucleus. PI-3K consists of a catalytic subunit, p110, and a regulatory subunit, p85, and there are 5 isoforms of each of the subunits. PI-3K phosphorylates Akt/PKB on serine and threonine residues which in turn modulates cellular processes like glycolysis and translation initiation and elongation (Burgering and Coffey, 1995). Akt/PKB also phosphorylates Bad, a pro-apoptotic protein. (Datta et al., 1997). When Bad is phosphorylated, it is sequestered by the 14-3-3 protein, rendering it incapable of

binding to the anti-apoptotic protein, bcl-2 which results in apoptosis. So Akt's phosphorylation of Bad serves to inhibit apoptosis and promote cell survival. This has deleterious effects for the organism because tumor cells are not permitted to undergo apoptosis and will survive and divide.

PI-3K has been linked to the development of colon cancer by a study showing that genetic inactivation of the p110 $\gamma$  catalytic subunit of PI-3K leads to the development of invasive colorectal adenocarcinomas in mice (Sasaki et al., 2000). This pathway is not completely separate from the Raf/MEK/ERK pathway as Akt has been found to inhibit Raf activity. In fact, one of the most interesting aspects of signal transduction is that few or perhaps no ras-mediated pathways operate completely independently; research is only beginning to identify the details of this crosstalk.

PI-3K is negatively regulated by the tumor suppressor gene, PTEN. PTEN, or MMAC (mutated in multiple advanced cancers) was first identified in the most aggressive form of brain cancer, glioblastoma multiforme. PTEN is mutated in a significant fraction of endometrial carcinomas, prostate carcinomas, and melanomas as well. PTEN is also associated with juvenile polyposis syndromes (Huang et al., 2000). PTEN's primary functions as a tumor suppressor gene are the induction of cell cycle arrest and apoptosis (Simpson and Parsons, 2001). PTEN is a dual-specificity phosphatase, meaning that it can dephosphorylate proteins at serine, threonine, and tyrosine residues. It specifically dephosphorylates PtdIns-3,4,5-P<sub>3</sub>, antagonizing the function of PI-3K. PTEN, therefore, acts as a negative regulator of Akt activation. Since

Akt can suppress apoptosis by the phosphorylation of the pro-apoptotic protein, Bad, PTEN can induce apoptosis of mutated or stressed cells to prevent tumor formation.

In addition to modulating apoptosis, PTEN plays a role in angiogenesis. PTEN suppresses the PI-3K-mediated induction of blood vessel growth factors like the vascular endothelial growth factor (VEGF) (Huang and Kontos, 2002). EGF and ras act to induce genes regulated by the hypoxia-induced factor (HIF-1), which is blocked by PTEN activity (Jiang et al., 2001). PTEN also inhibits cell migration and formation of focal adhesions when overexpressed in glioblastoma cell lines, suggesting that it helps to inhibit metastasis as well (Tamura et al., 1998).

PTEN also inhibits signaling from the insulin growth factor receptor (Nakashima et al., 2000). Insulin receptor substrates-1/2 (IRS-1/2) are docking proteins that are recruited by the insulin receptor and in turn, recruit PI-3K for signal transduction. The tumor suppressor function of PTEN helps to prevent aberrant signaling when insulin binds to its cell surface receptor.

Like PTEN, transforming growth factor (TGF)- $\beta$ 1 is another tumor suppressor gene that can signal in conjunction with ras. One study showed that TGF- $\beta$ 1 potentiates H-ras-induced COX-2 RNA stabilization (Sheng et al., 2000). TGF- $\beta$ 1 is unique as it can act as both an oncogene and tumor suppressor gene in a tissue-specific manner. TGF- $\beta$ 1 is growth stimulatory in endothelial cells but growth inhibitory for epithelial cells, rendering it a tumor suppressor gene in epithelial-derived cancers like colon cancer. The TGF- $\beta$  family of growth factors binds to 2 unique receptors, TGF- $\beta$  type I and type II. Tumor cells lose their response to the growth factor, and mutations in the receptors also

contribute to carcinogenesis. Ligand binding to the TGF- $\beta$  receptors causes intracellular signaling of other tumor suppressor genes, the SMADs (Moustakas et al., 2001). SMADs help to initiate TGF- $\beta$ -mediated gene transcription and are associated with juvenile polyposis (Roth et al., 1999) .

TGF- $\beta$ 1 normally inhibits growth of human colonic cells, but in the process of becoming tumorigenic, these cells obtain a decreased response to the growth inhibitory actions of TGF- $\beta$ . TGF- $\beta$ 1 also serves as an inhibitor of immune surveillance (Torre-Amione et al., 1990). TGF- $\beta$ 1 indirectly suppresses the function of the immune system by inhibiting the production of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and by inhibiting the expression of class II MHC molecules. TGF- $\beta$ 1 also promotes tumor progression by modulating processes necessary for metastasis (Markowitz et al., 1995), such as the degradation of extracellular matrix, tumor cell invasion and VEGF-mediated angiogenesis.

The TGF- $\beta$  receptor type II (T $\beta$ RII) is mutated in association with the MIN phenotype in most colorectal carcinomas (Markowitz et al., 1995). As many as 25% of colon cancers have missense mutations in the kinase domain of this receptor. A missense mutation in the kinase domain of the T $\beta$ RI has also been identified in metastatic breast cancer (Wakefield et al., 2001). It was also found that the expression of the TGF- $\beta$ II receptor is suppressed in metastatic oral squamous cell carcinomas compared to the primary tumor.

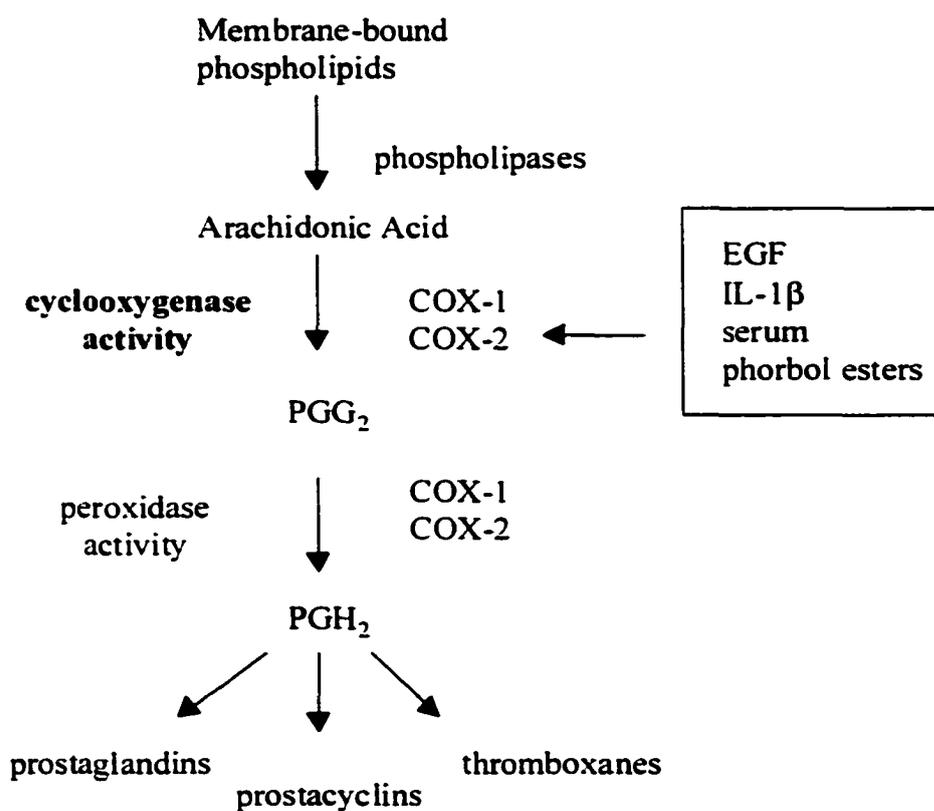
In contrast to APC mutations that occur early in colon carcinogenesis and K-ras mutations that occur in the promotion/progression phase, TGF- $\beta$  mutations occur at a later stage to promote metastasis and invasion. Mutations in the p53 tumor suppressor gene is another late stage event involved in the progression of carcinomas. p53 mutations are found in approximately 50% of colon carcinomas (Baker et al., 1990). The K-ras and p53 genes work together to prevent tumorigenesis in a normal cell. However, when p53 function is disrupted, the K-ras-mediated p19<sup>ARF</sup> expression does not result in its usual cell cycle arrest, but leads to increased cell proliferation (Brooks et al., 2001). Recent evidence proposes that the order of mutations may actually be opposite, with p53 mutations occurring at an earlier stage in tumorigenesis than K-ras (Hosaka et al., 2002). Further studies are needed to clarify the correct placement of p53 mutations in the genetic model of colon carcinogenesis, although their importance has been firmly established.

### **Arachidonic Acid Metabolism**

Cyclooxygenase (COX) enzymes catalyze prostaglandins from arachidonic acid (Figure 1). Arachidonic acid is released from the plasma membrane by phospholipases and is converted into prostaglandin G<sub>2</sub> (PGG<sub>2</sub>) by the COX enzymes. Arachidonic acid is also acted upon by the lipoxygenase (LOX) enzymes to form leukotrienes which are important for leukocyte movement in the inflammation process (Shureiqi and Lippman, 2001). The peroxidase activity of the COX enzymes then convert PGG<sub>2</sub> to PGH<sub>2</sub>, the parent compound for the rest of the prostaglandins. PGH<sub>2</sub> is converted into other prostaglandins, prostacyclins and thromboxanes, which are activated in a tissue-specific

manner. Prostaglandins play multiple roles in a variety of biological processes including blood clotting, ovulation, bone metabolism, nerve growth and development, and immune responses (Dubois et al., 1998). Prostaglandins bind to two classes of receptors, the G-protein coupled cytoplasmic EP1-4 receptors (Breyer and Breyer, 2000) as well as nuclear PPARs (peroxisome proliferator-activated receptors) which act as transcription factors (Forman et al., 1996).

There are two COX isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in most cell types and is necessary for homeostasis of colonic epithelium and platelet aggregation. COX-2, on the other hand, is undetectable in many normal adult tissues. It is an immediate early response gene inducible by a variety of stimuli such as growth factors, stress conditions and cytokines.



**Figure 1. Arachidonic acid metabolism - role of cyclooxygenases.** The cyclooxygenase enzymes are responsible for catalyzing prostaglandins from arachidonic acid. COX-1 is a constitutive enzyme. COX-2 is inducible by a variety of factors including growth factors, cytokines, serum and tumor promoters. Abbreviations: EGF, epidermal growth factor; IL-1 $\beta$ , interleukin-1 $\beta$ , PGG<sub>2</sub>/H<sub>2</sub>, prostaglandin G<sub>2</sub>/H<sub>2</sub>. Adapted from CS Williams and RN DuBois, *Am. J. Physiol.* 270:G393, 1996.

The COX-2 gene is 8.3 kb in size, is transcribed into 4.1 and 2.7 kb messages and results in a 74 kD protein. It is located on chromosome 1q25.2-q25.3 (Kosaka et al., 1994) and its gene product resides in the endoplasmic reticulum and the perinuclear envelope (Morita et al., 1995). It is unknown if COX-2 is one of the 15% of proteins that is translated in the nucleus (Iborra et al., 2001) but regardless, the prostaglandins it produces can permeate the nuclear membrane to easily target gene transcription. Inducible COX-2 expression has been studied in a variety of tissues, primarily mast cells of the immune system and epithelial-derived cancers.

COX-2 protein levels and prostaglandin production are upregulated in many tumor types, including pancreatic, gastric, breast, and skin cancers (Buckman et al., 1998; Hwang et al., 1998; Okami et al., 1999; Uefuji et al., 2000). COX-2 is also upregulated in 50% of colon adenomas and 85% of colonic carcinomas (Eberhart et al., 1994), suggesting that COX-2 influences both initiation and promotion/progression of colon tumorigenesis. COX-2 expression is found in stromal compartments of the normal colon and is increased in adenomas and carcinomas (Takahashi et al., 2000). Epithelial cells were positive for COX-2 staining in large, advanced carcinomas (Takahashi et al., 2000). Of course, it is also important to remember that COX-2 expression is found in the immune cells present in the inflammatory infiltrate near the tumor. It is still unclear in which cell type COX-2 protein most accumulates in colon tumors.

Several lines of evidence suggest that overexpression of COX-2 plays important roles in cell proliferation and colonic polyp formation as well as cancer progression. COX-2 upregulation leads to increased PGE<sub>2</sub> which has been shown to increase the

proliferation rate of human colon cancer cell lines (Qiao et al., 1995). Furthermore, selective pharmacological inhibition of COX-2 downregulates colon cancer cell growth (Sheng et al., 1997). COX-2 modulates metastatic potential by inducing matrix metalloproteinases, which can be directly inhibited by COX-2 inhibitors (Tsuji et al., 1997). In addition, cells overexpressing COX-2 secrete increased levels of angiogenic factors like VEGF and basic fibroblast growth factor (bFGF)(Tsuji et al., 1998).

COX-2 contributes to tumorigenesis not only by aiding in invasion but also by suppressing apoptosis of cancerous cells. PGE<sub>2</sub>, an end product in the cyclooxygenase reaction, has been shown to upregulate the anti-apoptotic protein, Bcl-2 when cells were treated with a COX-2-selective agent (Sheng et al., 1998) and also can induce caspase-3 cleavage (Takadera et al., 2002). NS398, a COX-2-specific inhibitor, induces apoptosis by releasing cytochrome c from the mitochondria, initiating the caspase cascade and culminating in the cleavage of PARP (Li et al., 2001). COX-2 also signals through Akt to promote cell survival (Hsu et al., 2000). Thus, COX-2 is considered to be an anti-apoptotic protein.

### **Bile Acids as Promoters of Colon Cancer**

Throughout the history of the study of colon cancer, it was believed that colon cancer was a consequence of poor diets. However, recent trials (Alberts et al., 2000) have challenged this belief and are placing more emphasis on genetic factors and their interaction with diet. A high-fat and low-fiber diet is generally regarded as a risk factor for colon cancer. Multiple studies have shown that people who consume Western diets consisting of more fat and meat have an increased risk for colon cancer compared to

those who eat diets low in fat and high in vegetables (Reddy and Wynder, 1973; Armstrong and Doll, 1975) (Cummings and Bingham, 1987; Kritchevsky, 1990). Thus, many interdisciplinary groups including basic scientists, epidemiologists, nutritionists and oncologists have sought to determine the underlying mechanisms of this observation.

One mechanism studied is the accumulation of bile acids in the feces. It was originally noted that those at high risk for colon cancer due to high fat diets also had elevated fecal bile acid levels compared to the healthy population. In addition, elevated fecal bile acid levels were observed in patients with colon adenomas and carcinomas (Reddy et al., 1977; Bayerdorffer et al., 1995).

Bile acids are polar derivatives of cholesterol and are a natural part of digestion. They are produced in the liver and excreted into the digestive tract to aid in the absorption and digestion of dietary fats (Borgstrom et al., 1985). However, in the case of high fat diets, bile acids can accumulate in the colon where enteric bacteria metabolize them to produce secondary bile acids (Everson and Jr., 1994). It is these secondary bile acids that are thought to increase the risk for colon cancer. There are four main bile acids - two primary bile acids that do not affect colon cancer risk and two secondary bile acids which do. The primary bile acids are cholic and chenodeoxycholic and the secondary bile acids are lithocholic and deoxycholic acids. Deoxycholic acid has been the one most studied as contributing to colon carcinogenesis.

The exact function of deoxycholic acid (DCA) in colon tumorigenesis is not yet known but many possible mechanisms have been proposed. Firstly, when biopsies from the human ascending and descending colon were incubated with DCA, the colonic crypt

cells entered a hyperproliferative state; in fact, the upper crypt labeling was 30-52% higher after DCA incubation (Bartram et al., 1993). These growth changes in the cells lining the colonic crypts are considered to be one of the initiating steps towards colon tumorigenesis.

In addition to changes in cell proliferation, DCA has been implicated in signal transduction. Secondary bile acids can activate protein kinase C, a stress induced kinase that can be regulated by the K-ras oncogene (Craven et al., 1987; Huang et al., 1992). This stimulation is thought to occur by increasing levels of the second messenger, diacylglycerol (DAG). It is important to note that the levels of some PKC isozymes are increased and some decreased in carcinogen-induced colonic tumors (Wali et al., 1995), suggesting a yet unidentified link between gene expression and dietary factors in the process of colon carcinogenesis. The main biological effect of DCA is the induction of apoptosis (Marchetti et al., 1997). DCA-mediated apoptosis has been reported to involve the Fas receptor (Qiao et al., 2001).

Carcinogen-treated rodent models of colon tumorigenesis have been widely used to study the mechanism of bile acids. Carcinogen-induced rats fed diets containing bile acids presented with higher tumor incidence than those treated with carcinogen only (Cohen et al., 1980; Morvay et al., 1989). These studies suggested that DCA was acting as a tumor promoter. Bile acids are unable to induce tumors alone, verifying that they are neither initiators nor complete carcinogens in animal models.

Since elevated fecal bile acids are obtained through the preventable means of diet, this surrogate endpoint marker for colon cancer has been under intensive study as a target

of chemoprevention. Ursodeoxycholic acid (UDCA) was shown to be a chemopreventive agent in two models of carcinogen-induced rat models (Earnest et al., 1994) (Narisawa et al., 1998; Narisawa et al., 1999). This compound also causes growth arrest in human colon cancer cells (Powell et al., 2001) and can inhibit apoptosis by preventing cytochrome c release (Rodrigues et al., 1999). UDCA was first used as a treatment for gallstones and then for cholestatic liver diseases. But since it has been shown to inhibit DCA-induced apoptosis (Rodrigues et al., 1998), UDCA is now in clinical trials for the prevention of polyp recurrence.

### **Chemoprevention - NSAIDs**

The COX enzymes have also come under intensive study as a target for colon cancer prevention. Several epidemiological studies have linked prolonged use of nonsteroidal anti-inflammatory drugs (NSAIDs) to a 40-50% reduction in mortality from colon cancer (Kune et al., 1988; Rosenberg et al., 1991; Thun et al., 1991; Pelage et al., 1994). The primary mechanism of NSAIDs is the inhibition of COX enzyme activity. The NSAIDs, aspirin, piroxicam and sulindac, just to name a few, have shown efficacy in reducing the number and size of carcinogen-induced colon tumors in animal models (DuBois et al., 1996). However, inhibition of COX-1 leads to undesirable side effects. Since COX-1 protein is important for the maintenance of the GI tract, its inhibition causes ulceration, bleeding, and perforation. This is due to the lack of necessary prostaglandins to protect the GI lining and the stomach from harsh, acidic conditions. Renal toxicity is also associated with chronic intake of NSAIDs.

To circumvent some of these negative side effects, a new class of NSAIDs was developed: COX-2 selective inhibitors. The creation of these compounds was made possible by the finding that COX-1 and COX-2 enzymes have different structural binding sites for the NSAIDs (Rowlinson et al., 1999). Multiple studies have illustrated that COX-2-selective inhibitors suppress tumorigenesis in multiple intestinal neoplasia (Min) mice. COX-2 selective drugs were almost as effective at inhibiting intestinal polyps in APC<sup>Δ716</sup> mice as crossing them with COX-2 knockout mice (Oshima et al., 1996). COX-2 selective inhibitors also suppress tumor cell growth in nude and SCID mice (Koki et al., 1999). The same phenomena has been shown in human chemoprevention trials. In fact, the COX-2 selective drug, celecoxib, was recently passed by the FDA for treatment of FAP patients.

There are currently two COX-2 selective drugs that are commercially available with FDA approval, Celecoxib and Vioxx. Both are presently prescribed to alleviate pain and inflammation associated with rheumatoid arthritis. Both are also actively being pursued as chemopreventive agents for a variety of cancers. Their main mechanism of action is the suppression of COX-2, but not COX-1 enzyme activity, and they have a multitude of other effects stemming from this inhibition. Celecoxib, for example, induces apoptosis in prostate cancer cells by blocking the Akt survival pathway (Hsu et al., 2000). Celecoxib is also effective at inhibiting the process of angiogenesis (Masferrer et al., 2000).

NSAIDs exert their anti-cancer effects by several different mechanisms, including the inhibition of cyclooxygenase enzyme activity and promotion of apoptosis. The

chemopreventive drug, Sulindac is a prime example of multiple mechanisms of action. Sulindac has two metabolites, Sulindac sulfide and Sulindac sulfone. Sulindac sulfide is a potent inhibitor of COX enzyme activity and induces apoptosis (Piazza et al., 1995). Sulindac sulfone (Exisulind), on the other hand, inhibits COX-2 protein expression, linking its effects to signal transduction (Taylor et al., 2000). An example of sulfone's effect on signaling is that it can inhibit cyclic guanosine monophosphate phosphodiesterase V (cGMP-PDE V), allowing protein kinase G activation and apoptosis (Thompson et al., 2000). This apoptosis cascade involves reduction of free  $\beta$ -catenin levels and the activation of c-jun N-terminal kinase (JNK) and extracellular regulated kinase (ERK) (Soh et al., 2000).

Thus, NSAIDs have both COX-dependent and COX-independent mechanisms of action. Several studies have hinted at the possibility of other cellular targets of NSAIDs. For example, COX-2 null colon cancer cells are growth-inhibited by NSAIDs just as much as cells expressing COX-2 (Hanif et al., 1996; Elder et al., 1997). In addition, prostaglandins produced by the COX enzymes cannot rescue cells from NSAIDs-induced apoptosis (Narisawa et al., 1984; Chan et al., 1998).

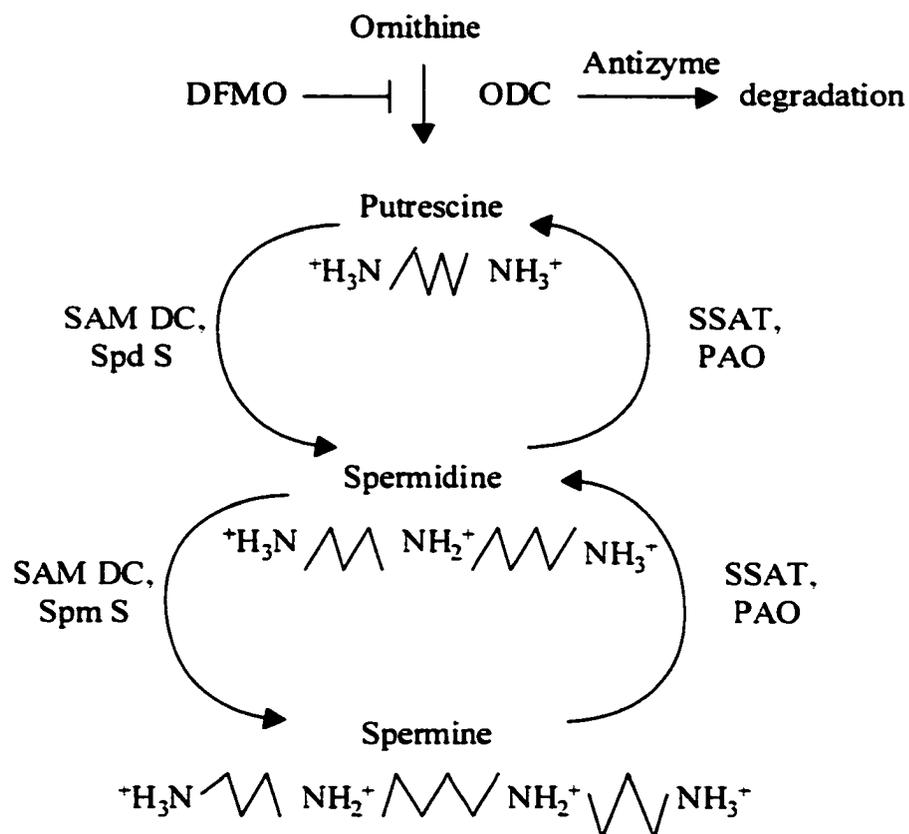
It has become evident that these COX-independent mechanisms of action may be equally important to the anti-cancer effects as the ability to inhibit cyclooxygenases. The newest signaling pathway identified to be a target of NSAIDs is that of nuclear factor- $\kappa$ B (NF- $\kappa$ B) (Kopp and Ghosh, 1994). Sulindac is the NSAID that has been shown to inhibit the activation of NF- $\kappa$ B (Yamamoto et al., 1999). Another COX-independent mechanism involves peroxisome proliferator activator receptors (PPARs). The NSAID sulindac,

inhibits DNA binding ability of PPAR $\delta$  to its responsive elements (He et al., 1999), leading to an abrogation of the carcinogenic process. PPAR $\delta$  has been reported to be an APC-dependent gene but this signaling is COX-independent.

### **Polyamine Metabolism and Cellular Functions**

Polyamines are ubiquitous small cations found in prokaryotes and every living cell in eukaryotes, plants and animals. In mammalian cells, the most abundant polyamines are putrescine, spermidine and spermine. At physiological pH, the amine groups are protonated, and putrescine is a divalent, spermidine is a trivalent, and spermine is a tetravalent cation (Meyskens and Gerner, 1999). They are found in near millimolar concentrations in human cells and tumors.

Polyamine pools are tightly regulated by the interaction and cooperation of several enzymes (Figure 2). Ornithine decarboxylase is the first biosynthetic enzyme in polyamine metabolism and this enzyme makes putrescine from its ornithine substrate. Ornithine is originally catalyzed from arginine pools by the arginase enzyme. Putrescine is then converted into spermidine by S-adenosyl methionine decarboxylase (SAMDC) and spermidine synthase. Aminobutyl groups are again added on to make spermine from spermidine by the enzymes SAMDC and spermine synthase.



**Figure 2. Polyamine Metabolism.** Ornithine is the substrate used by ODC to make putrescine which is then catalyzed into spermidine and spermine. The polyamines are catabolized by SSAT and PAO. Antizyme binds to ODC to target it for degradation by the 26S proteasome. Abbreviations: SAMDC, S-adenosylmethionine decarboxylase, Spd/Spm S, spermidine/spermine synthase; SSAT, Spermidine/spermine N<sup>1</sup>-acetyl transferase; PAO, FAD-dependent polyamine oxidase.

ODC activity is regulated by yet another enzyme called the anti-enzyme for ODC or more simply, antizyme (AZ). As its name suggests, antizyme is a negative regulator of ODC and thus, its production is dependent on an increase in polyamines. Antizyme production is unique because it is contingent upon a novel translational frameshifting mechanism. Of the two overlapping open reading frames (ORFs) that encode antizyme, only the first has a usable AUG initiation codon. And yet the second ORF encodes most of the protein. Thus, translation begins at the AUG in the first ORF. Under conditions of high polyamine contents, the ribosome stalls at the stop codon, frameshifts, either in the +1 or -2 frame to the stop codon in the second ORF and continues translation (Matsufuji et al., 1996). Frameshifting is strictly dependent on a unique pseudoknot structure in the antizyme RNA. The efficiency of frameshifting is dependent upon polyamine pools and thus, an increase in polyamines by ODC will result in an increase in frameshifting of antizyme in order to keep ODC in check.

The method by which antizyme keeps ODC in check is by targeting it for degradation by the proteasome. ODC is active only as a homodimer. Antizyme has high affinity to ODC monomers and thus binds to ODC to prevent homodimerization (Everson and Jr., 1994). Two antizyme molecules convert one ODC homodimer into two inactive AZ-ODC monomers. Antizyme then targets these ODC monomers to the proteasome for degradation without requiring ubiquitination of the protein first (Murakami et al., 1992). ODC is the only known protein degraded by the proteasome in an ubiquitin-independent manner. Once ODC is in the proteasome, it is completely destroyed, while antizyme is

recycled in order to participate in several rounds of ODC degradation (Everson and Jr., 1994).

The catabolism of polyamines is also orchestrated by tightly regulated enzyme activity. The enzyme N<sup>1</sup>-spermidine/spermine acetyl transferase (SSAT) acetylates spermidine and spermine. Acetylated spermine is then cleaved into spermidine by the flavin adenine dinucleotide (FAD)-dependent polyamine oxidase. The same reaction occurs to catabolize spermidine into putrescine.

Just as the metabolism of polyamines is regulated in multi-step enzymatic reactions, there are several transport mechanisms that allow polyamines in and out of the cell (Seiler et al., 1996). However, these mechanisms are not fully identified nor defined. Polyamines may enter the cell by multiple transporters, which are independent of amino acid transporters (Kakinuma et al., 1988). Two main mechanisms have been suggested for polyamine uptake: Na<sup>+</sup>-dependent and Na<sup>+</sup>-independent (Byers et al., 1987; Byers and Pegg, 1989). Cell surface heparan sulfate proteoglycans have been shown to facilitate polyamine uptake (Belting et al., 1999).

Polyamines are shuttled out of the cell by a diamine exporter that functions to rid cells of the diamines putrescine, cadavarine and monoacetylated spermidine (Xie et al., 1997). This diamine exporter has only been characterized biochemically and specific information about the precise mechanism of this exporter is still missing. Most recently, two studies suggest the coordination of heparan sulfate proteoglycans and polyamines at the cell surface in mediating proliferation and tumorigenesis (Ding et al., 2001; Belting et al., 2002).

The ubiquitous expression of polyamines in multiple cell types has led to the discovery of their many and varied functions. Polyamines are involved in embryonic development, cell growth and cell cycle regulation, apoptosis and carcinogenesis. Several lines of evidence show a role for polyamines in development. First, the specific pharmacological inhibitor of ODC, DL- $\alpha$ -difluoromethylornithine (DFMO), can terminate pregnancies in rodents (Reddy and Rukmini, 1981; O'Toole et al., 1989). *C. elegans* expressing a mutant ODC gene can grow in medium containing polyamines but showed developmental deficiencies in polyamine-free medium (MacRae et al., 1998). In addition, ODC has recently been reported to be essential for cell survival in early murine development (Pendeville et al., 2001).

It is not surprising that molecules affecting fetal development are also involved in cell proliferation. Polyamines appear necessary for growth and progression through the cell cycle. Putrescine levels are elevated in the S and G2 phases, spermine increases in G1 and S phases, while spermidine levels remain high throughout the cell cycle (Fredlund et al., 1995). In addition, polyamines interact with cell cycle regulating proteins. Polyamines are required for the degradation of cyclin B1 (Thomas and Thomas, 1994) and are important in maintaining levels of cyclin D1 and preventing cell cycle arrest in G1 (Hong et al., 1998).

Polyamines play a more promiscuous role in cell death than in cell proliferation. Polyamines can both be protective as well as stimulatory for apoptosis. Firstly, polyamines have been reported to prevent both ionomycin-induced apoptosis in T cells and dexamethasone-induced apoptosis in thymocytes (Brune et al., 1991; Desiderio et al.,

1995). Spermine's role as a free radical scavenger is slowly being elucidated as well (Ha et al., 1998). In contrast, overexpression of ODC can drive accelerated cell death in IL-3-deprived myeloid cells (Packham and Cleveland, 1994). Products from polyamine catabolism also potentiate apoptosis. Spermine and spermidine are oxidized by polyamine oxidase (PAO) as well as serum amine oxidases and these reactions leave behind strong inducers of apoptosis like hydrogen peroxide (Ha et al., 1997). Thus, it appears that polyamines play both antagonistic and protagonistic roles in cell death. Depending on the cellular environment, the signals a cell receives and the levels of cellular stress, polyamines can function as protectors or effectors of apoptosis.

Considering that polyamines are influential in cell proliferation and apoptosis, the door is open for polyamines to drive the process of carcinogenesis. Polyamines are generally believed to be carcinogenic molecules. Polyamine pools are greater in neoplastic cells and tissues than their normal counterparts (Porter et al., 1987). The overexpression of ODC in NIH3T3 cells caused neoplastic transformation in the first study to propose that ODC is an oncogene (Moshier et al., 1993). ODC expression can also act as a tumor initiator and promoter in mouse skin (Megosh et al., 1995; O'Brien et al., 1997). ODC can cooperate with H-ras mutations to promote skin tumorigenesis (Smith et al., 1998). In addition to cooperating with oncogenes, polyamines are affected by tumor suppressor genes. Our laboratory group has shown that wild-type APC can downregulate ODC expression in human colon cancer cells (Fultz and Gerner, 2002).

Overproduction of polyamines have been documented in many different cancer types including breast, cervical, prostate, skin and colon cancers. Thus, polyamine

metabolism has become a target of chemoprevention. DFMO, the specific inhibitor of ODC activity, inhibits cancer cell growth. DFMO inhibits putrescine and spermidine pools without affecting spermine levels (Meyskens and Gerner, 1999), except in the prostate (Simoneau et al., 2001). Human cancer cells treated with DFMO stop proliferating and this phenotype can be rescued by exogenous polyamines, suggesting that DFMO has a specific effect on polyamine biosynthesis as opposed to nonspecific cytotoxic effects. DFMO has proven to be efficacious in animal models of bladder, breast, and colon tumorigenesis (Nigro et al., 1986; Nowels et al., 1986; Thompson and Ronan, 1986; Erdman et al., 1999).

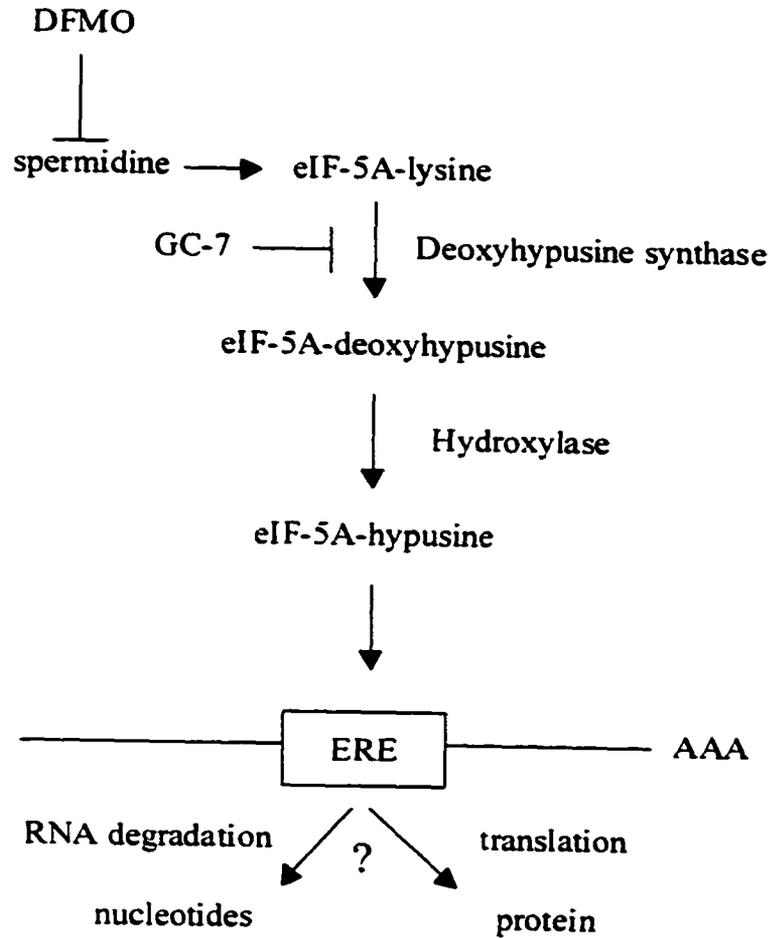
DFMO has been a promising chemopreventive agent in clinical trials. Although dose de-escalation was necessary due to ototoxicity, low levels of the drug are now in trials for breast, Barrett's esophagus, cervical, and prostate cancers (Meyskens and Gerner, 1999). DFMO is also being studied in combination with the NSAID sulindac for colon cancer prevention (Meyskens and Gerner, 1999).

### **Eukaryotic Initiation Factor (eIF)-5A**

Polyamines also influence RNA structure and function. A prime example of this is the role of the polyamine-dependent eukaryotic initiation factor (eIF)-5A. eIF-5A is actually not considered an initiator of translation, even though it was originally found to influence translation and named for that role. eIF-5A was isolated from the ribosome-bound fraction and can stimulate the synthesis of methionyl puromycin (Benne et al., 1978; Smit-McBride et al., 1989). However, inhibition of eIF-5A does not lead to complete abolishment of general protein synthesis, suggesting other roles for eIF-5A in

the process of making protein from RNA (Duncan and Hershey, 1986; Kang and Hershey, 1994). eIF-5A has been implicated in RNA processing, but its exact role is still unknown (Zuk and Jacobson, 1998).

eIF-5A is a very unique protein in that it is the only known protein to require a hypusine modification for functionality (Park et al., 1981). When eIF-5A is translated, it contains a lysine residue (Park et al., 1982) that is then modified posttranslationally by two distinct enzymes into the functional eIF-5A protein, containing a hypusine residue (Figure 3). Since the first enzymatic step is contingent upon spermidine levels, eIF-5A is described as a polyamine-dependent protein (Chen, 1983). The first enzyme involved is deoxyhypusine synthase, which catalyzes the transfer of a 4-aminobutyl group from spermidine to the lysine residue to make eIF-5A-deoxyhypusine. Then the activity of deoxyhypusine hydroxylase completes the process, culminating in eIF-5A-hypusine. It is this form of eIF-5A that is the active protein that will interact with RNA. eIF-5A protein binds to the eIF-5A response element (ERE) with the sequence, AAATGTCACAC (Xu and Chen, 2001), turning on specific gene expression.



**Figure 3. eIF-5A modification and function.** Spermidine pools are necessary for the modification of eIF-5A-lysine to the catalytically active eIF-5A-hypusine. The deoxyhypusine synthase and hydroxylase enzymes work to form eIF-5A which then binds to the eIF-5A response element (ERE) on RNA to mediate either RNA degradation or translation into protein. GC-7 is a specific inhibitor of deoxyhypusine synthase.

Knockouts of eIF-5A or deoxyhypusine synthase result in a lethal phenotype in yeast (Park et al., 1998). In addition, eIF-5A and hypusine appear to be necessary for mammalian cell growth, a similar scenario to the polyamines (Park et al., 1993; Chen and Liu, 1997). The fact that this protein so crucial for cell proliferation requires two distinct enzymatic steps to become viable suggests a need to keep its cellular levels in check. And yet it appears to be working on the most transient of nucleic acids, RNA. Determining the function of this protein and how it may contribute to carcinogenesis seems incredibly beneficial.

Thus far, it is determined that eIF-5A interacts with a variety of nucleocytoplasmic shuttling proteins, including CRM1 and the HIV protein, Rev. eIF-5A has been identified as a binding partner with Rev, a gene necessary for the viral RNA export of HIV (Schatz et al., 1998). Since this RNA is shuttled out of the nucleus for translation, it was postulated from this data that eIF-5A was part of a protein-protein complex that functions as a pore or a pump on the nuclear membrane. A similar finding was described for eIF-5A interaction with the export receptor, CRM1 (Rosorius et al., 1999). CRM1 is an essential exporter of proteins, such as NF- $\kappa$ B, containing nuclear export signals (NESs) (Wen et al., 1995). It is also interesting to note that Rev has a NES. Thus, eIF-5A may be a protein that shuttles between the nucleus and the cytoplasm. It is unknown what determines its localization at a particular time, although it seems to be at least partially dependent on the relative amounts of accessible binding partners.

### **Statement of the Problem**

Colon cancer incidence is influenced by a number of specific genetic and intestinal luminal risk factors. The inducible cyclooxygenase-2 (COX-2) is found to be upregulated in 50% of small colonic adenomas and increases in expression throughout the promotion/progression phases of carcinogenesis. COX-2 could be an important pathophysiological target for the prevention or treatment of colorectal and other cancers. Chemopreventive drugs which inhibit COX-2 are under current investigation in clinical studies evaluating their ability to suppress colon carcinogenesis. The mechanisms by which genetic and intestinal luminal risk factors influence COX-2 expression are still not well understood.

Hypothesis: Mutations in the APC tumor suppressor gene and the K-ras oncogene and increases in luminal risk factors, like secondary bile acids and polyamines, positively and negatively regulate COX-2 expression in human colon cancer cells.

### Specific Aims:

1. Characterize the signaling pathways by which K-ras activation induces COX-2 and wild-type APC suppresses COX-2. Stable transfections of K-ras or APC or both in human colon cancer cells will be used to study the importance of PKC- and PI-3K-dependent signaling in COX-2 expression.

2. Determine the effects of deoxycholic acid on COX-2 expression in cells with K-ras and APC mutations. DCA-mediated COX-2 transcription will be investigated using promoter-reporter constructs.
3. Determine if the chemopreventive agent, Sulindac, and its metabolites, can suppress K-ras-mediated induction of COX-2 expression.
4. Investigate the possible influence of polyamine synthesis on COX-2 expression in colon cancer cells.

## **CHAPTER 2: MATERIALS AND METHODS**

### **Cell Culture and Treatments**

Caco-2, HT-29, HCT-116 cells were obtained from ATCC (Rockville, MD). All cell culture reagents were purchased from Invitrogen Corp. (Carlsbad, CA). Caco-2 cells were maintained in MEM, 10% fetal bovine serum, 1% penicillin/streptomycin. HT-29 and HCT-116 cells were grown in McCoy's 5a medium, supplemented with 10% FBS and 1% pen/strep. HT29-APC and HT29- $\beta$ Gal cells were obtained from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD) and were maintained in McCoy's 5a supplemented with 10% FBS and 1% pen/strep and 0.6 mg/ml hygromycin B for selection (Morin et al., 1996). The HT29-APC cell line has an inducible wild-type APC gene under the control of a metallothionein promoter. Thus, addition of  $\text{ZnCl}_2$  to the medium induces wild-type APC in the HT29-APC cells; the HT29- $\beta$ Gal cells serve as a negative control for that cell line because they have a nonfunctional  $\beta$ -Gal gene in place of the APC gene. Both cell lines were treated with 300  $\mu\text{M}$   $\text{ZnCl}_2$  before subsequent experiments were conducted. HCA-7 cells were obtained from Dr. David Alberts (University of Arizona, Tucson, AZ) and were grown in DMEM supplemented with glutamine, 10% FBS, 1% pen/strep.

All inhibitory or stimulatory agents were solubilized at 100-1000X concentrated stocks and then diluted into medium upon the cells. Sulindac, sulindac sulfide and

sulfone were purchased from ICN Biochemicals, Inc., Aurora, OH, and obtained as a generous gift from GA Piazza (Cell Pathways, Inc., Horsham, PA). Previous work from our lab indicated that the dose to reduce colony formation by 50% (IC<sub>50</sub> dose) in both parental and K-ras transfected clones was 120  $\mu$ M for sulindac sulfide, 600  $\mu$ M for sulindac sulfone, and 400  $\mu$ M for sulindac sulfoxide. DMSO served as the vehicle.

LY 294002, deoxycholic acid, cycloheximide, and putrescine were purchased from Sigma. Calphostin C and TPA were purchased from Calbiochem (La Jolla, CA). DMSO was used as the vehicle for LY294002, TPA, Calphostin C and water was used a vehicle for the rest of the aforementioned compounds.

DFMO, was a generous gift from ILEX Oncology, San Antonio, Texas. N<sup>1</sup>-guanyl-1,7-diaminoheptane (GC-7) was a generous gift from Dr. Myong Hee Park (National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD).

## **Gene Transfection Experiments**

### ***Stable transfection of the K-ras oncogene***

K-ras cDNA was purchased from ATCC and ligated into the multiple cloning site of a pCDNA3 mammalian expression vector (Invitrogen) containing a CMV promoter and a neomycin resistance gene (Lawson et al., 2000). The pcDNA3-Kras plasmid was transfected into Caco-2 cells using the calcium phosphate transfection technique as described in the literature (Ausubel, 1995). The K-ras activated clones were maintained under selection with 350  $\mu$ g/ml G418. The pcDNA3-Kras plasmid was transfected into the HT29-APC and HT29- $\beta$ Gal cells using the lipofectamine reagent (Invitrogen) and the

cells were maintained in 0.6 mg/ml hygromycin B and 350 µg/ml G418 for selection of wild-type APC and activated K-ras, respectively.

K-ras activation was confirmed by a PCR-RFLP method. Caco-2 cells and K-ras activated clones were lysed in buffer containing 100 mM NaCl, 10 mM Tris HCl pH 8, 25 mM EDTA, 0.5% SDS, 100 µg/ml proteinase K and incubated at 37°C for 16 hours. DNA was isolated with two extractions in phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated on ice in 3 M Na acetate and ethanol. The pellet was washed in 70% ethanol and further purified in 100 µg/ml RNase, 0.5% SDS, 100 µg/ml proteinase K. Another phenol:chloroform:isoamyl alcohol extraction was performed, the DNA pellet was precipitated with ethanol and then resuspended in TE buffer. DNA concentration was determined by UV spectrophotometric methods. PCR was performed with specific primers (Invitrogen) to create a BstNI restriction site at codon 12 of the human K-ras gene. The upstream primer sequence used was 5': AAA CTT GTG GTA GTT GGA CCT, the downstream primer was 5': TTG TTG GAT CAT ATT CGT CC. Mutation at codon 12 of K-ras alters the BstNI site, preventing restriction enzyme cleavage. PCR products were run out on a 4% NuSieve:agarose (3:1) gel stained with ethidium bromide.

#### ***Transient Transfections of Luciferase Reporter Constructs***

The human COX-2 reporter constructs (-1432/+59, -327/+59, -220/+59, -124/+59, -52/+59 ) was obtained from Dr. Hiroyasu Inoue (National Cardiovascular Center Research Institute, Osaka, Japan) (Inoue et al., 1994). Cells were plated at  $0.3 \times 10^6$  cells/well on 6-well plates 24 h prior to transfection. Cells were transfected with 1 µg DNA/well using lipofectAMINE reagent (Invitrogen) per the manufacturer's instructions

and incubated in complete medium for 24 h prior to luciferase assay. In addition to the COX-2 promoter constructs, cells were cotransfected with a CMV- $\beta$ Gal construct and  $\beta$ -gal assays (Promega, Madison, WI) were performed to control for transfection efficiency.  $\beta$ -Gal activity was normalized to protein concentration. The error bars indicate  $n=3$ . The mutant p53 plasmid (Arg273His) was a generous gift from Dr. Jesse Martinez, University of Arizona and was transfected using the same procedure outlined above.

COX-2 reporter constructs were also made for the 3'-UTR to study mRNA stability. The parent luciferase construct was made by excising the luciferase gene from the pGL3 plasmid (Promega) and ligating it into pcDNA3.1(+) (Invitrogen) at the Xba I and Hind III sites. The 3'UTR of COX-2 was amplified by PCR from Caco-2 cells using the sense primer 5'-TCTAGACAGAAGTCAGTACTCCTGTTGC-3' and the antisense primer 5'-TCTAGAGCACCTACTGAATTGGCTTC-3'. These primers put a Xba I cut site on each end of the PCR product. The pcDNA-luciferase plasmid was then cut at Xba I and the COX-2 3'-UTR was ligated in (Dixon et al., 2000; Sheng et al., 2000). The proper orientation of the insert was verified by diagnostic cuts with Pst I.

### **Western Blotting and Immunoprecipitations**

Subconfluent cells were lysed on ice in ripa buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 30  $\mu$ g/ml aprotinin, 100  $\mu$ M sodium orthovanadate, 10  $\mu$ g/ml PMSF) and electrophoresed on 10-15% SDS-PAGE gels, depending on the protein size. Proteins were transferred to nitrocellulose membrane. Membranes were blocked in Blotto A, probed with the appropriate antibodies and developed by the ECL chemiluminescence system (Amersham, Arlington Heights, IL). The COX-2 antibody was obtained from

Santa Cruz Biotechnology (Santa Cruz, CA) and used at a 1:5000 dilution. Blots were stripped and reprobed with  $\beta$ -actin (Sigma, St. Louis, MO) antibody as a loading control. All western blots shown are a representative of 3 or more experiments unless otherwise noted.

The AZ1-Gst plasmid used for immunoprecipitation experiments was made by a former student in the lab, Xiaozhen Xie (Ref: Xie dissertation, 1996). Bacterial cultures were begun from glycerol stocks and grown overnight. The cultures were then stimulated with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) for 4 hours. The cells were spun down at 7000 x g for 7 min and the pellet was lysed with STE buffer (0.1 M NaCl, 10 mM Tris pH 8, 1 mM EDTA) containing 100  $\mu$ g/ml lysozyme. The samples were incubated on ice for 5 min and then adjusted to 5 mM DTT and 1.5% sarcosyl. The lysate was sonicated twice for 30 sec. The lysate was then run through Gst purification columns (GSTrap-Fast Flow, Amersham) and wash procedures were followed per manufacturer's instructions. The  $A_{280}$  value of the Gst-tagged protein was determined and protein concentration was estimated using the ratio  $A_{280} \sim 1, 0.5$  mg/ml Gst-tagged protein. Human cell lysate or purified ODC protein (Ning Qu, Gerner lab) was then incubated overnight with Gst-AZ1 protein and 400  $\mu$ l of 50% glutathione-agarose slurry (Sigma, catalog # G4510) on a rotator at 4°C.

The samples were spun at 2500 rpm for 5 min at 4°C and the supernatant was gently pipetted off. The pellet was washed four times with ice-cold PBS. The pellet was resuspended in 30  $\mu$ l SDS-PAGE loading buffer, boiled for 5 min, and then loaded onto a

10% SDS-PAGE gel. Cell lysate or recombinant protein was also run on the gel to serve as a positive control. Western blots were then performed for ODC and COX-2.

### **Northern Blotting**

RNA was isolated from frozen cell pellets using Trizol:chloroform (5:1) (Invitrogen) extractions. RNA was further purified in isopropanol and washed in 75% ethanol. RNA was run out on a 1% agarose/formaldehyde gel in MOPS buffer and transferred to nylon. The COX-2 cDNA probe (Oxford Biomedical Research, Inc., Oxford, MI) was labeled using the RTS RadPrime DNA labeling system (Gibco, Rockville, MD) or the Decaprime II labeling system (Ambion, Austin, TX) and [ $\alpha^{32}\text{P}$ ]-dCTP. The probe was purified with G-50 Sepharose columns (Boehinger Mannheim, Indianapolis, ID) and quantitated with a scintillation counter. The membrane was hybridized to the probe at  $1 \times 10^7$  cpm overnight at 42°C and then washed 3 times (2X SSC/0.1% SDS for 5 min. at RT and then for 20 min. at RT, 0.5X SSC/0.1% SDS for 30 min. at 65°C). Glyceraldehyde-3-phosphate dehydrogenase (0.75 kb *PstI-XbaI* fragment) was used as a loading control. Autoradiograms were quantitated by densitometric analysis (ImageQuant, Molecular Dynamics, Sunnyvale, CA). All northern blots shown are a representative of 2 or more experiments unless otherwise noted.

### **Prostaglandin E<sub>2</sub> Determination**

Cells were seeded  $10^4$ /well on 96-well plates and treated with drug or vehicle 24 hours later. Serum-free medium was supplemented with 15  $\mu\text{M}$  arachidonic acid (Sigma)

after 24 hours of drug treatment for 1 h prior to medium collection for the PGE<sub>2</sub> kit (Amersham).

### **Tumorigenicity Assay**

Caco-2 or K-ras activated Caco-2 cells (clone 60) were injected subdermally into 4 areas of the flank of SCID mice at  $1 \times 10^6$  cells/injection. There were 4 mice per group. The mice were fed 167 ppm sulindac in AIN93G diet (Teklad, Indianapolis, IN). The injection sites were monitored 1x/week until tumors appeared; tumors were measured 2x/week. The animals were sacrificed at 100 days due to tumor burden.

### **<sup>3</sup>H-Polyamine Incorporation Experiments**

For <sup>3</sup>H-putrescine uptake experiments, cells were plated at  $3 \times 10^6$  cells/100mm plate 24 h before addition of 1-15  $\mu$ Ci/ml <sup>3</sup>H-putrescine. At various timepoints, the cells were trypsinized and washed twice with ice cold PBS. The cell pellet was resuspended in 200  $\mu$ l PBS and 150  $\mu$ l was added to 10 ml of scintillation fluid. The cpm values were normalized to protein concentration. The samples were done in triplicate.

### **RNA Gel Shifts**

The three RNA probes of the eIF5A sequences in the COX-2 3'UTR were made by PCR and *in vitro* transcription. The sense primer was that for T7 polymerase (TAATACGACTCACTATAGGG) to prime the sequence for the *in vitro* transcription kit. The sequence for ERE #1 was 5'-

TAATACGACTCACTATAGGGAAATGCCAAAT

TTATTAATTTGGTGGAGCCACT-3', the sequence for ERE #2 was 5'-  
 TAATACGACTCACTATAGGGTCATTCACACATTAATTTTATCTCAGTCTTGA  
 AGCCAATTC-3', and the sequence for ARE/ERE was 5'-  
 TAATACGACTCACTATAGG  
 GATGTCACTACTCTA AAGATTTTGCTGTTGCTGTTAAG-3'. The antisense primer  
 for ERE #1 was 5'-AGTGGCTCCACCACCTTAAT-3', for ERE #2 was 5'-  
 GAATTGGCTTCAAGAC-3', for ARE/ERE was 5'-CTTAACAGCAACAGC-3'. The  
 DNA was amplified and the salts were purified out using Microcon columns (Millipore,  
 Bedford, MA). The DNA was then boiled for 5 min and placed on ice for 10 min to undo  
 any secondary structure that might interfere with transcription. The Promega T7 *in vitro*  
 transcription kit was used, following the manufacturer's protocol. The cold RNA  
 products were run out on a 1% agarose/formaldehyde gel in MOPS buffer to check for  
 proper size. The <sup>32</sup>P-labeled probes were purified with G-25 Sepharose columns and  
 quantitated with a scintillation counter.

Nuclear extracts were prepared from cell pellets from  $1 \times 10^7$  Caco-pcDNA or  
 Caco-Kras cells. The pellets were resuspended in 5 volumes of hypotonic buffer (10 mM  
 HEPES pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT) and then  
 centrifuged at 3400 rpm for 15 min at 4°C. The pellet was resuspended in 3 volumes of  
 hypotonic buffer and swelled on ice for 3.5 h until 90% of cells were lysed as visualized  
 by trypan blue. Samples were centrifuged at 4500 rpm for 15 min at 4°C. The supernatant  
 was saved as the cytosolic extract and the pelleted nuclei were resuspended in a half  
 volume of low salt buffer (20 mM HEPES pH 7.9, 25% glycerol, 1.5 mM MgCl<sub>2</sub>, 0.02 M

KCl, 0.2 mM EDTA, 0.2 mM PMSF and 0.5 mM DTT). In a flat bottom test-tube containing a stir bar, a half volume of high salt buffer (low salt buffer with 1.2 M KCl) was added dropwise and kept stirring on ice for 30 min. Nuclei were spun at max speed for 30 min @ 4°C and the supernatant saved for protein determination.

Whole cell extracts (10-20 µg, lysed in ripa buffer) or nuclear extracts of Caco-pcDNA and Caco-Kras cells were incubated for 10 min at RT with Dialysis Buffer (20 mM HEPES pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF and 0.5 mM DTT) and dI-C. Then the radioactive probe +/- the cold probe were added to the mixture for 30 min at RT. The samples were run out on a 5% acrylamide gel and shifts were determined by autoradiography.

### **Polyamine Analysis**

5-10 x 10<sup>6</sup> cells were trypsinized as usual and washed 2x in PBS. The cell pellet was resuspended in 0.1 M HCl at a concentration of 5 x 10<sup>6</sup> cells/900 µl. On ice, the samples were sonicated for 10 sec. The mixture was adjusted to 0.2 M HClO<sub>4</sub> and incubated overnight at 4°C. The samples were centrifuged for 7 min at 2000 rpm at 4°C. The supernatant was used for polyamine analysis via high-performance liquid chromatography (HPLC). The HPLC column was a µBondapak C18 reverse phase column (Milipore-Waters, Milford, MA) and the polyamines were eluted, derivatized, and detected (Seiler and Knodgen, 1980). The pellet was resuspended in 0.5 M NaOH overnight and the protein concentration was determined with the detergent compatible D<sub>c</sub> protein assay (Biorad, Hercules, CA). Extracellular polyamines were measured by

concentrating the polyamines in a minimal amount of media for 1-4 hours and then the media was harvested as the cell pellets above. All polyamine values reported were repeated 2 or more times.

### **ODC Activity Assay**

Trypsinized cells were washed in PBS and spun down at 8000 rpm. The cell pellets were resuspended in ODC buffer (50 mM Na buffer, 0.1 mM EDTA, 1 mM DTT, 50  $\mu$ M pyridol phosphate, 0.1 mM PMSF) at  $2.5 \times 10^6$  cell/100  $\mu$ l and sonicated for 10 sec on ice. Samples (200  $\mu$ l) were placed at the bottom of a 15-ml conical tube with  $^{14}$ C-ornithine and cold ornithine for competition. The tubes were covered with specialized rubber stoppers which held a filter paper containing NCS solubilizer. The samples were incubated at 37°C for 30 min. A syringe and needle were used to add 1 M citric acid to each sample and the CO<sub>2</sub> was captured by the filter overnight at RT. The filter paper was counted using a scintillation counter and normalized to protein concentration. Samples were done in triplicate.

### **Statistical Analysis**

Assessment of statistical differences was determined by ANOVA. A  $P < 0.05$  was considered statistically significant.

## **CHAPTER 3:**

### **K-RAS- AND APC-DEPENDENT SIGNALING OF COX-2**

#### **Introduction**

COX-2 is upregulated in animal models of colon carcinogenesis, including the AOM-induced rat (DuBois et al., 1996) and the Min mouse model of FAP (Boobol et al., 1996; Williams et al., 1996). COX-2 is also more highly expressed in human colon carcinomas than normal adjacent mucosa (Eberhart et al., 1994). Multiple pieces of clinical evidence suggest that the inhibition of COX-2 with nonsteroidal anti-inflammatory drugs (NSAIDs) leads to a suppression of colon cancer risk and mortality (Kune et al., 1988; Rosenberg et al., 1991; Thun et al., 1991; Pelage et al., 1994). Thus, COX-2 appears to play a significant role in the development of colon cancer.

K-ras mutations are found in 47% of carcinomas (Vogelstein et al., 1988) and APC mutations in 80-90% (Miyoshi et al., 1992; Iwamoto et al., 2000). Both K-ras and APC have been reported to modulate COX-2 levels in a variety of cell culture and animal models. Neither K-ras nor APC affect COX-2 transcription; both genes influence COX-2 posttranscriptionally. Ras activation can stabilize COX-2 mRNA in rat intestinal epithelial cells, thereby increasing COX-2 protein levels and enzyme activity. K-ras stabilization can be mediated by the serine-threonine kinase Akt/PKB (Sheng et al., 2001) and H-ras-induced stabilization by TGF- $\beta$  (Sheng et al., 2000). Ras-mediated signaling of COX-2 transcription has been reported to work through MAPK activation (Sheng et al.,

1998) and PKC-mediated events (Zhang et al., 1998). The induction of wild-type APC downregulates COX-2 protein expression without affecting mRNA steady levels in human colon cancer-derived HT-29 cells (Hsi et al., 1999). Both a null mutation for COX-2 or treatment with a COX-2 inhibitor in APC<sup>Δ716</sup> mice, a model for FAP, dramatically reduces the size and number of polyps (Oshima et al., 1996; Oshima et al., 2001).

In these experiments, the role of K-ras-mediated and APC-mediated signaling on COX-2 expression was examined in human colon cancer cells. Several K-ras mediated pathways were investigated by pharmacological interventions, using COX-2 transcriptional and translational regulation as endpoints. Two human colorectal adenocarcinoma-derived cell lines, Caco-2 and HT-29, have normal K-ras and mutant APC genes. These cells were either stably transfected with a constitutively activated K-ras oncogene or an inducible full-length APC gene or both in order to elucidate the mechanisms by which their gene products function to control COX-2 expression. The data indicate that APC and K-ras regulate COX-2 by distinct posttranscriptional mechanisms in human colon cancer-derived cell lines and that the relative importance of these mechanisms is cell line specific.

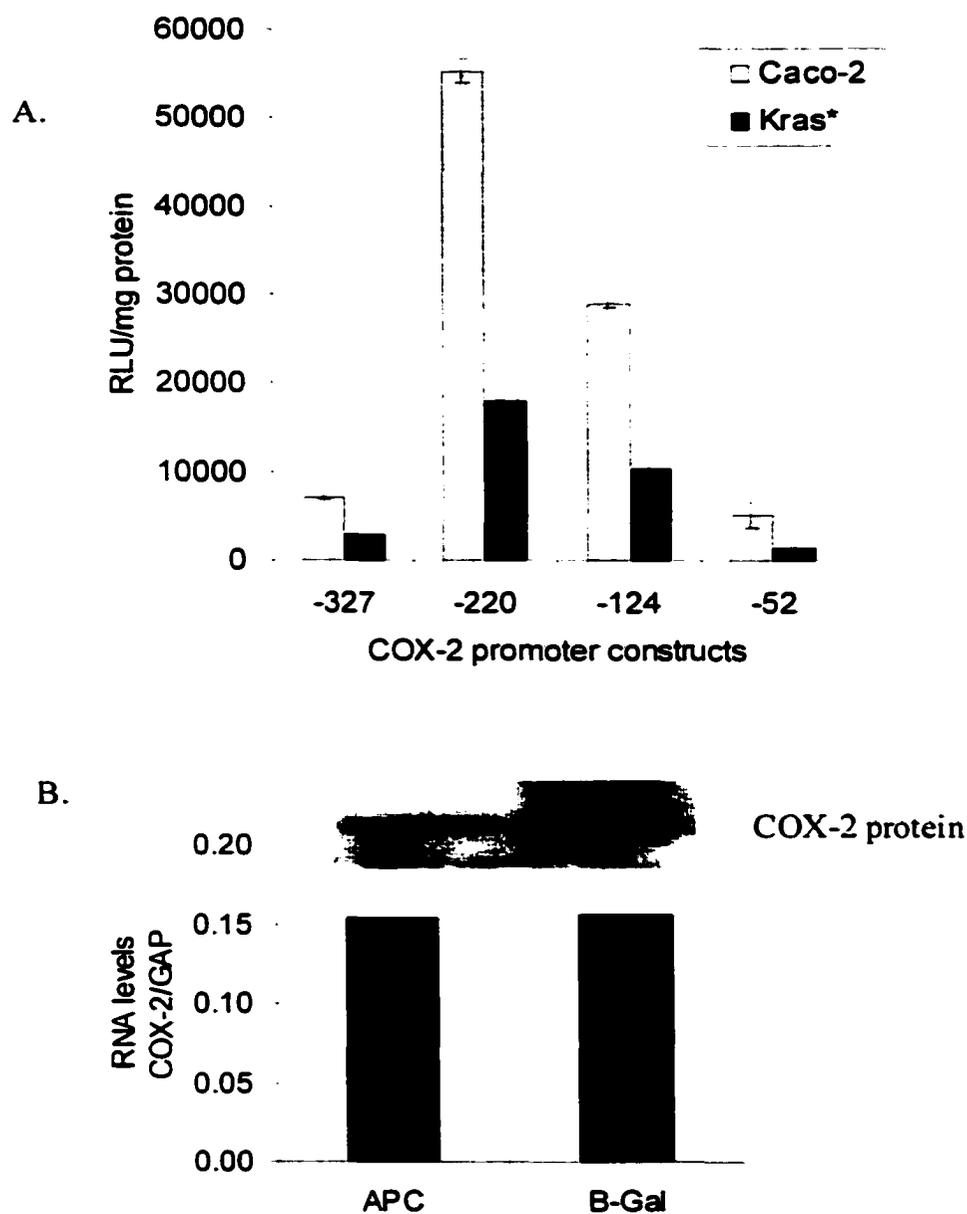
## **Results**

### ***K-ras and APC mediate COX-2 expression by posttranscriptional mechanisms***

We have previously published that K-ras activation upregulates COX-2 mRNA and protein expression (Taylor et al., 2000). To determine if this is due to an increase in transcription, COX-2 promoter-reporter constructs were transfected into the Caco-2 and

K-ras activated Caco-2 cells. K-ras caused a significant decrease in promoter activity compared to the Caco-2 cells in all four constructs tested (Figure 4A), even though data in chapter 5 shows upregulation of COX-2 message and protein (Figure 16). This data suggests that the upregulation of COX-2 RNA is not transcriptionally-mediated.

APC has also been reported to influence COX-2 protein levels (Hsi et al., 1999). Using the HT29-APC and HT-29 $\beta$ Gal cells, we verified that APC regulates COX-2 posttranscriptionally. Wild-type APC induced by ZnCl<sub>2</sub> addition to the media caused a decrease in COX-2 protein without affecting RNA levels in the HT29-APC cells (Figure 4B). There was no effect of ZnCl<sub>2</sub> addition in the HT29- $\beta$ Gal cells. ZnCl<sub>2</sub>-induced APC protein downregulation was time-dependent, with a significant decrease obtained by 6 hours of treatment.

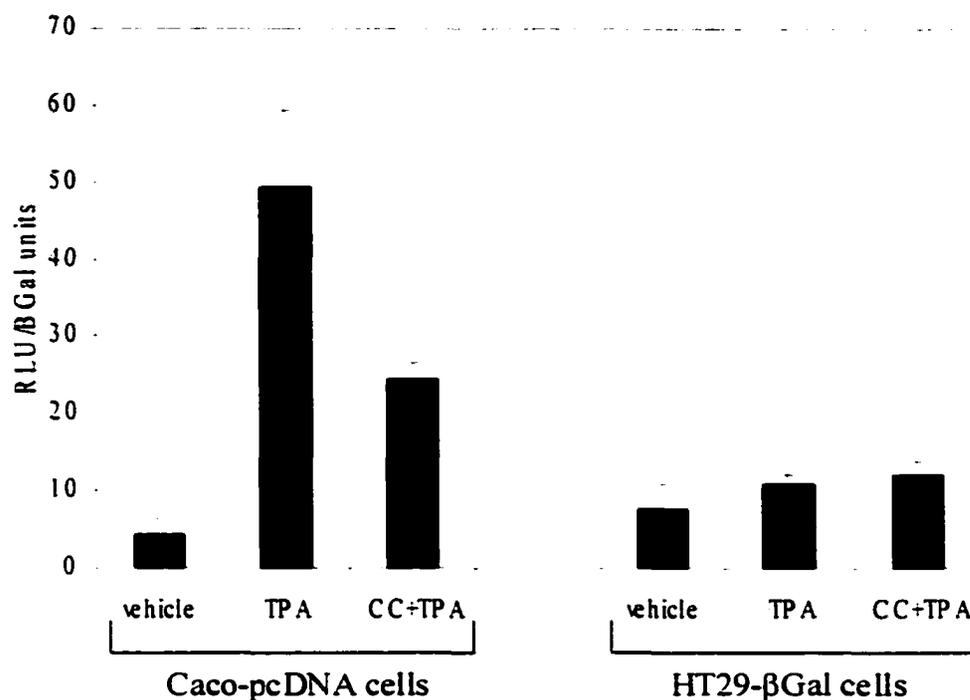


**Figure 4. K-ras and APC regulate COX-2 posttranscriptionally.** A. COX-2 promoter-reporter constructs were transiently transfected into Caco-2 and K-ras activated (\*) Caco-2 cells. Luciferase activity was normalized to protein concentration. The error bars indicate triplicate samples. B. HT29-APC and HT29- $\beta$ Gal cells were treated with  $ZnCl_2$  for 6 h and were harvested for COX-2 western blot analysis (above) and COX-2 northern blot analysis (below). COX-2 RNA levels were normalized to GAPDH levels to control for loading.

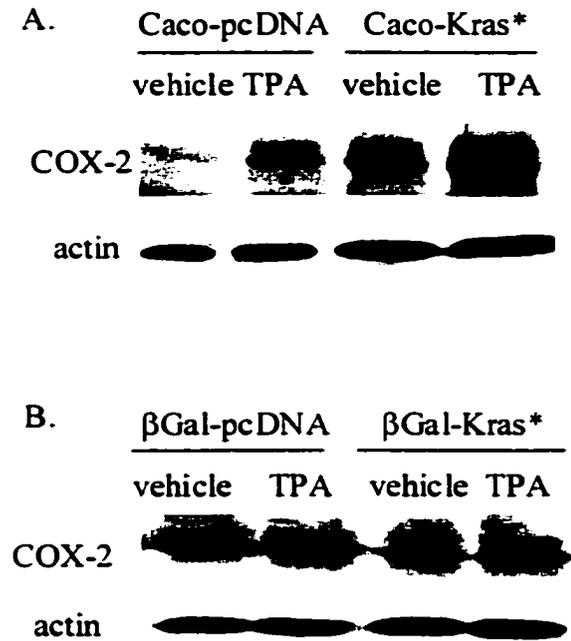
### ***Protein kinase C-mediated regulation of COX-2 transcription***

The role of K-ras dependent PKC activation was studied relative to COX-2 promoter activity. PKC is a family of serine-threonine protein kinases that can influence the proliferation, differentiation, and rate of apoptosis of colonic epithelial cells (Rickard et al., 2000). 12-O-tetradecanoylphorbol-13-acetate (TPA), an activator of PKC, has been shown to stimulate COX-2 transcription in a variety of cell model systems. The colon cancer cell lines in this study reacted differently to PKC modulation. TPA significantly induced COX-2 promoter activity as well as protein expression in the Caco-2 and the K-ras activated Caco-2 cells (Figures 5, 6A). However, TPA was not able to induce either COX-2 promoter activity or protein expression in the HT-29 (HT29- $\beta$ Gal) cells (Figures 5, 6B).

The Caco-2, K-ras activated Caco-2, and HT29- $\beta$ Gal cells all have a mutant APC gene so this discrepancy in TPA-mediated COX-2 expression is probably not APC-dependent. The induction of COX-2 promoter activity seen with treatment of TPA in the Caco-2 cells was found to be PKC-specific. This is illustrated in Figure 5 where transiently transfected cells were pretreated with the PKC inhibitor, Calphostin C, for 1 hour prior to stimulation with TPA. This pretreatment inhibited the TPA-mediated induction of COX-2 promoter activity by 50%, verifying that this is indeed a PKC-dependent phenomenon. This data, along with the insensitivity to TPA, suggests that HT-29 cells may have deregulated PKC signaling.



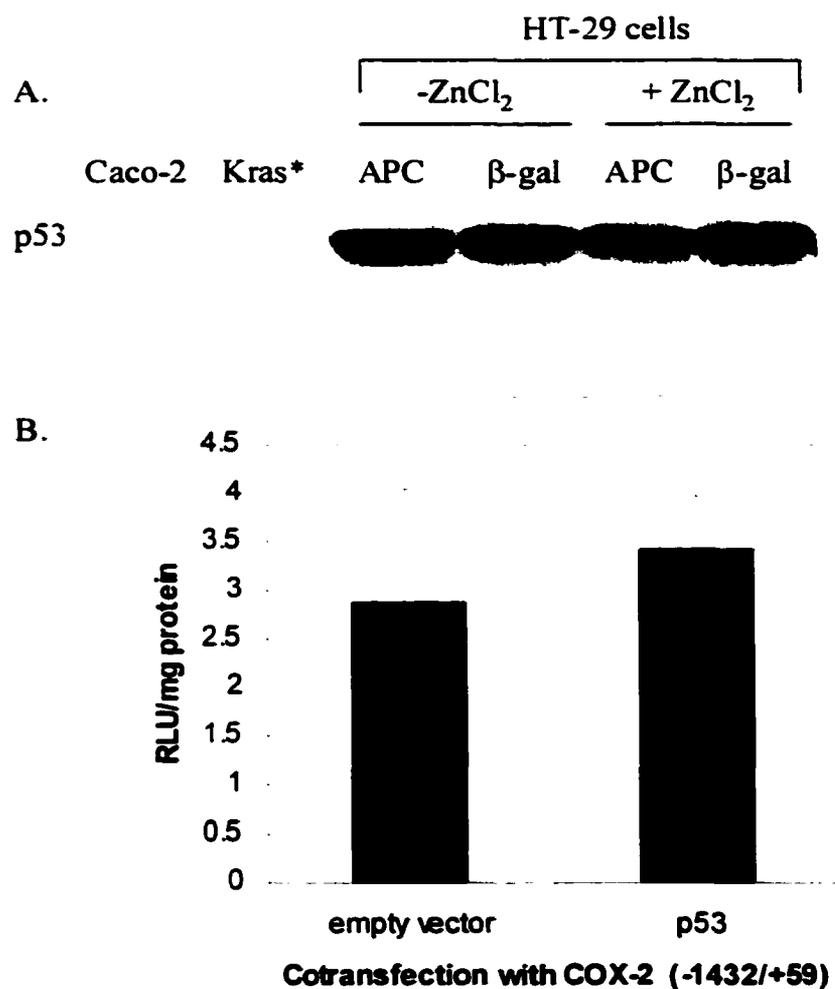
**Figure 5. Activation of PKC leads to cell-type specific effects on COX-2 promoter activity.** Caco-pcDNA or HT29-βGal cells were transiently transfected with the COX-2 promoter construct (-1432/+59) and then pretreated with 200 nM Calphostin C (CC) or DMSO vehicle for 1 h before treatment with 50 ng/ml TPA or DMSO vehicle for 24 h. Luciferase activity was normalized to β-Gal activity and protein concentration. Stastical analysis was performed by one-way ANOVA and significance was found for Caco-pcDNA cells: vehicle-treated cells compared to TPA-treated cells ( $p < 0.004$ ) and for TPA-treated cells compared to CC+TPA-treated cells ( $p < 0.02$ ).



**Figure 6. Effects of PKC activation on COX-2 protein expression.** A. COX-2 western blot analysis of TPA-stimulated Caco-pcDNA and K-ras activated Caco-2 cells. B. COX-2 western blot analysis of TPA-stimulated HT29βGal-pcDNA and K-ras activated HT29-βGal cells in the absence of ZnCl<sub>2</sub>. Actin protein expression was used as a loading control.

Since Caco-2 and HT-29 cells have the same APC and K-ras mutational status, we decided to look into a possible role for p53 in this discrepancy in PKC signaling. p53 is mutated in both cell lines but the HT-29 cells express high levels of this mutant protein, while Caco-2 cells do not express any mutant p53 protein (Figure 7A). Perhaps the overexpression of mutant p53 was somehow preventing PKC signaling in the HT-29 cells. Or conversely, the lack of p53 expression was allowing for PKC signaling in the Caco-2 cells. There are several instances in the literature where PKC and p53 are interdependent. For example, p53 protein phosphorylation is inversely dependent upon PKC activation (Nakamura et al., 2000). In addition, PKC can influence p53 transcriptional activity and phosphorylation (Youmell et al., 1998). PKC also cooperates with p53 in growth arrest of ras-transformed fibroblasts (Delphin and Baudier, 1994). And finally, there is evidence that p53 can regulate COX-2 transcription (Subbaramaiah et al., 1999). So, we hypothesized that p53 could be interfering with PKC signaling of COX-2.

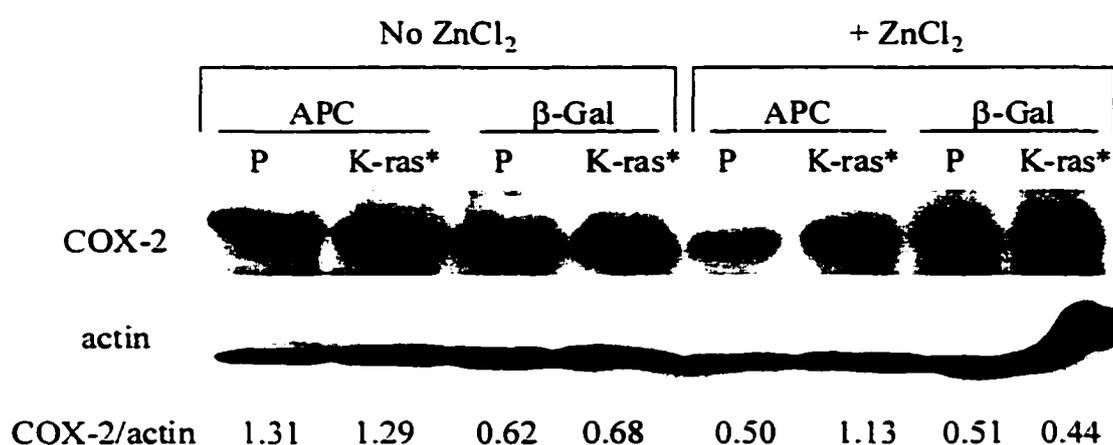
A construct containing the mutant p53 found in HT-29 cells (Arg273His) was transiently transfected into Caco-2 cells along with the largest available COX-2 promoter construct. It was hypothesized that exogenous mutant p53 expression would downregulate promoter activity in the Caco-2 cells if the overexpression of p53 was the reason for lack of PKC signaling in the HT-29 cells. However, the mutant p53 expression plasmid had no significant effect on COX-2 promoter activity in the Caco-2 cells, suggesting that overexpression of mutant p53 was not the reason for inhibition of PKC signaling in the HT-29 cells (Figure 7B). This data also shows that APC has no effect on p53 protein expression in HT-29 cells (Figure 7B).



**Figure 7. p53 protein expression differs in Caco-2 and HT-29 cell lines.** A. Western blot analysis of both normal and mutant p53 in the Caco-2, K-ras activated Caco-2, HT29-APC and HT29-βGal cells in the presence or absence of ZnCl<sub>2</sub>. B. Caco-2 cells were transiently cotransfected with a mutant p53 (arg273his) expression plasmid and the -1432/+59 COX-2 promoter construct. Luciferase activity was normalized to protein concentration.

***Activated K-ras does not induce COX-2 Expression in HT-29 cells***

In the process of studying PKC signaling, it became clear that the regulation of COX-2 in HT-29 cells is very different than in the Caco-2 cell model. To explore this further, an activated K-ras was stably transfected into the HT29-APC and HT29- $\beta$ Gal cells to investigate a possible interaction between APC and K-ras in COX-2 regulation. An activated ras was confirmed with K-ras PCR-RLFP and K-ras western blot (data not shown). It was previously established in our lab and others that ras activation induced COX-2 protein (Sheng et al., 1998; Taylor et al., 2000). However, K-ras activation did not increase COX-2 expression levels in the HT29- $\beta$ Gal cell line (Figure 8). In contrast, K-ras was able to induce COX-2 in the HT29-APC cell line. This was an unusual finding in the sense that the Caco-2 cell line is most similar to the HT29- $\beta$ Gal cell line in mutational status; they both have a mutant APC. Thus, APC is probably not the reason for the distinct effects of K-ras in these two cell lines. APC may be able to overcome this aberrant signaling as wild-type APC allows K-ras to mediate increased levels of COX-2 protein. This mechanism may be jointly PKC- and APC-dependent. PKC may suppress the K-ras-mediated COX-2 induction while wild-type APC is able to restore the induction in this cell line.



**Figure 8. K-ras activation does not induce COX-2 protein expression in HT-29 cells with a mutant APC.** HT-29 cells were stably transfected with an empty pcDNA3 plasmid or an activated K-ras oncogene (p21<sup>Val12</sup>). Several clones were selected for their increased expression of K-ras protein and K-ras mutational status as seen by PCR-RFLP analysis. HT29APC-pcDNA, HT29APC-Kras, HT29βGal-pcDNA, and HT29βGal-Kras cells in the presence or absence of ZnCl<sub>2</sub> (6 h treatment) were analyzed for COX-2 protein expression by western blot. Actin protein served as a loading control.

***Wild-type APC inhibits PI-3K-dependent signaling of COX-2 protein***

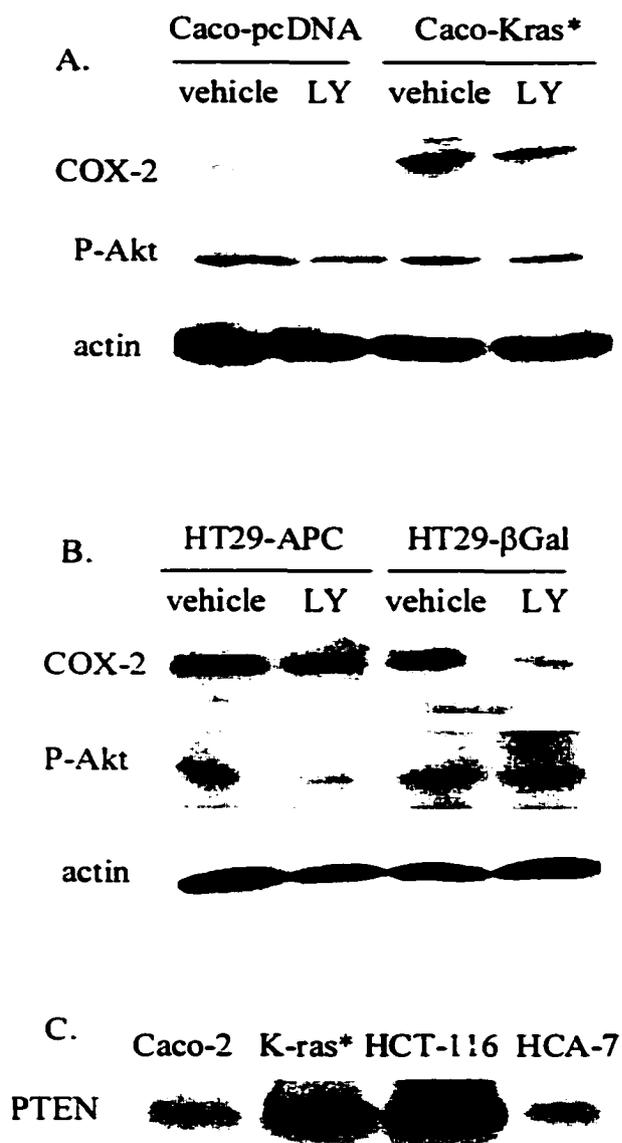
PI-3K is a downstream target of ras and continues through multiple signaling pathways to stimulate cell survival (Rodriguez-Viciana et al., 1994). PI-3K directly activates Akt/PKB which leads to the activation or inactivation of apoptosis-related genes like Bad and NF- $\kappa$ B (Datta et al., 1997; Romashkova and Makarov, 1999). Recent studies have reported that a dominant negative form of Akt interferes with constitutive COX-2 expression (Oshima et al., 2001), and K-ras-dependent induction of COX-2 in rat intestinal epithelial cells is dependent upon the activation of Akt (Sheng et al., 2001). One mechanism of action of celecoxib, a selective COX-2 inhibitor, is the inhibition of Akt activation in human prostate cancer cells harboring a K-ras mutation (Hsu et al., 2000). Thus, a relationship between Akt, activated ras and COX-2 has been established. However, when we treated Caco-2 and K-ras activated Caco-2 cells with the PI-3K inhibitor, LY294002, we saw a reduction of COX-2 protein in both cell lines, suggesting a K-ras-independent pathway in the PI-3K/Akt-dependent signaling of COX-2 (Figure 9A). The concentration of LY294002 that partially inhibits COX-2 also partially inhibits phospho-Akt protein as seen in Figure 9A, which shows that LY294002 does indeed disrupt downstream signaling of PI-3K. However, the decrease in COX-2 protein appears more pronounced than the decrease in phospho-Akt, suggesting that the inhibition of PI-3K is causing downregulation of COX-2 protein by Akt-dependent and -independent mechanisms. Perhaps K-ras is directly activating Akt, independently of PI-3K in rat intestinal epithelial cells, while in Caco-2 cells, the pathway is mediated upstream by PI-3K.

Although Akt appears to modulate COX-2 by direct K-ras-dependent (Sheng et al., 2001) and indirect K-ras-dependent mechanisms, the role of APC in PI-3K/Akt-mediated signaling of COX-2 has not yet been established. However, it is known that the expression of the catalytic subunit of PI-3K, p110 $\gamma$ , can affect growth of colon cancer cells with mutations in APC (Sasaki et al., 2000). In this study, it is shown that a wild-type APC interferes with the PI-3K-dependent signaling of COX-2 protein. Wild-type APC has been reported to inhibit COX-2 at the translational level (Hsi et al., 1999) so we investigated the effects of PI-3K suppression on COX-2 protein expression in cells with both wild-type and mutant APC. Treatment of HT29- $\beta$ Gal cells with LY294002 causes a significant reduction in the COX-2 protein levels (Figure 9B) as seen in both Caco-2 cell lines, which also have a mutant APC gene. However, when full-length wild-type APC is induced by ZnCl<sub>2</sub> treatment, followed by suppression of PI-3K by LY294002, there is no apparent change in COX-2 protein (Figure 9B). Thus, one mechanism of APC modulation of COX-2 translation may be through the inhibition of PI-3K signaling.

Since the PTEN tumor suppressor gene inhibits PI-3K activity, and multiple tumor suppressor genes (i.e. APC, p53) are known to modulate COX-2 expression, we hypothesized that PTEN could regulate COX-2. PTEN protein levels were established for some of the colon cancer cell lines used in this study. First, it is interesting to note that cells with mutant K-ras, Caco-Kras and HCT-116, express higher levels of PTEN protein as seen by western blot (Figure 9C). If K-ras plays a role in PTEN expression it would probably be a PI-3K-independent mechanism since K-ras did not influence COX-2 levels modulated by the PI-3K inhibitor (Figure 9A). Also, there seems to be only a small effect

of the activated K-ras gene in the Caco-2 cell model. However, once again, the cells with the activated ras oncogene express higher amounts of PTEN protein. This suggests that PTEN expression can be induced by an activated K-ras, in a cell-specific manner.

Also, the levels of PTEN appear to be inversely proportional to COX-2 protein expression in the RER+ cell lines, HCT-116 and HCA-7. HCT-116 cells, which have no detectable COX-2 protein, have the highest levels of PTEN expression, while HCA-7 cells, which constitutively express COX-2, have the lowest levels of the cell lines tested. This argument does not hold true of cells derived from adenocarcinomas, like the Caco-2 cell model. The Caco-Kras cells express more of both COX-2 and PTEN than the parental line. The potential role of APC in PTEN expression is currently being studied.



**Figure 9. PI-3K-dependent signaling of COX-2.** A. COX-2, phospho-Akt (473Ser) and actin westerns were performed on lysates made from Caco-pcDNA and Caco-Kras cells treated with 10  $\mu$ M LY294002 for 24 h. B. After 6 h of ZnCl<sub>2</sub> pretreatment, HT29-APC and HT29-βGal cells were treated with 20  $\mu$ M LY294002 for 24 h and lysed for COX-2, phospho-Akt and actin westerns blots. C. Western blot analysis of normal and mutant PTEN protein expression in Caco-2, K-ras activated Caco-2, HCT-116 and HCA-7 cells.

## Summary

We and others have begun to characterize the role of several signaling pathways contributing to COX-2 expression in colon tumorigenesis, including the downstream signaling of K-ras and APC. Both K-ras and APC mutations regulate COX-2 posttranscriptionally. This study shows that this posttranscriptional regulation is cell line specific, since K-ras mediates COX-2 expression differently in Caco-2 compared to HT-29 cells. This cell line specificity may be due to signaling stemming from protein kinase C. In Caco-2 and K-ras activated Caco-2 cells, stimulation of PKC by TPA results in an induction of COX-2 transcription. However, TPA yields little to no effect on COX-2 in HT29- $\beta$ Gal cells. These data suggest that HT-29 cells are TPA-insensitive and signal COX-2 through a PKC-independent mechanism. It is interesting to note that Caco-2 cells are nontumorigenic (Figure 18) and HT-29 cells are tumorigenic when injected into immune-compromised mice (personal communication, MA Nelson, University of Arizona). Perhaps PKC is a determining factor in the tumorigenic potential of these cell lines.

An activated K-ras oncogene also signals COX-2 differently in the Caco-2 cells compared to the HT-29 cells. Two previous studies (Sheng et al., 1998; Taylor et al., 2000) have shown that activated ras genes result in an upregulation of COX-2 expression in colon cancer cell lines. K-ras activation yields increased COX-2 mRNA steady state levels independent of promoter activity in Caco-2 cells (Figure 4). Also, H-ras promotes COX-2 mRNA stability through an AU-rich region in the 3'-UTR which translates into greater amounts of COX-2 protein in rat intestinal epithelial cells (Sheng et al., 2000).

However, our experiments revealed that an activated K-ras transfected into the HT29- $\beta$ Gal cells does not lead to an increase in COX-2 expression as is seen in the Caco-2 and rat intestinal epithelial cell models (Figure 8). This cell type specificity could be due to expression of other genes in the HT-29 cells which are able to modulate COX-2 expression, irrespective of K-ras mutational status. These genes may be able to cooperate with APC since overexpression of a mutant K-ras in the presence of a wild-type APC does allow for an upregulation of COX-2 expression in the HT-29 cells. This data suggests that wild-type APC may be able to relieve the suppression of PKC-dependent signaling of COX-2 in this cell line.

APC also mediated PI-3K-dependent signaling in this study. The suppression of the PI-3K/Akt pathway in cells with a mutant APC resulted in an inhibition of COX-2 protein expression; this effect was observed in the Caco-2, K-ras activated Caco-2, and the HT29- $\beta$ Gal cells. Cells that express a full-length APC gene, the HT29-APC cell model, did not exhibit this reduction in COX-2 protein levels when treated with the PI-3K inhibitor. Thus, wild-type APC seems to deregulate PI-3K-dependent signaling of COX-2. Since this interference in the PI-3K-dependent signaling of COX-2 is conserved in both the Caco-2 and HT-29 cell lines, this pathway may serve a more important role in COX-2 regulation than other pathways that signal COX-2 in only one cell line.

The dependence of PI-3K-mediated COX-2 expression on APC may be due to a reliance on the activation of Akt. An activated Akt can phosphorylate GSK-3 $\beta$  (Pap and Cooper, 1998), a protein involved in the APC/ $\beta$ -catenin complex. When this complex is deregulated,  $\beta$ -catenin translocates into the nucleus to induce Tcf/Lef transcriptional

activation of genes like c-myc. As COX-2 is a downstream target of APC signaling, it may also be influenced by the proper formation of APC in its complex with GSK-3 $\beta$ . This type of protein-protein interaction could be disrupted by the inhibition of PI-3K by LY294002 and thus, could result in an inhibition of COX-2 expression. In contrast, it may be that PI-3K is interfering with APC-mediated COX-2 expression. Our data does not determine whether APC is upstream of PI-3K, or vice versa.

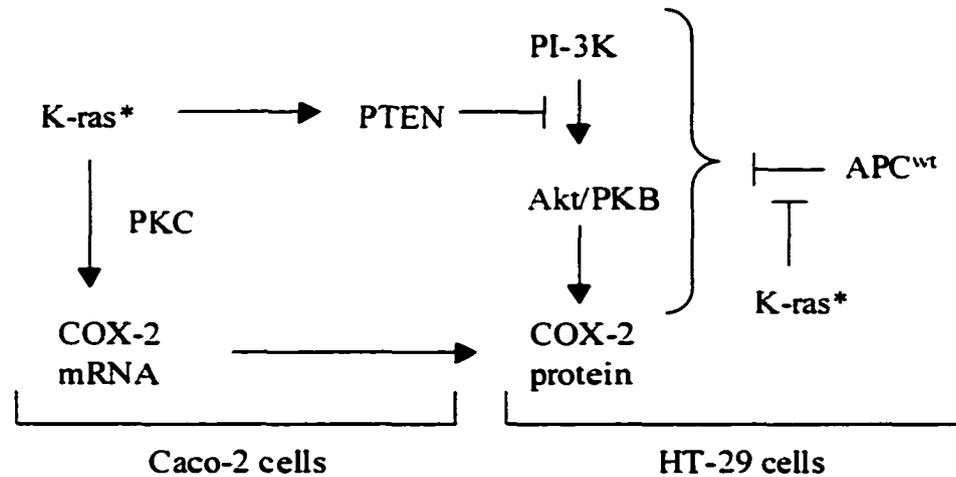
In light of the APC-dependent modulation of PI-3K in the HT-29 cells and the cell-type specific regulation of PTEN expression, we are currently pursuing a possible interaction between the PTEN and APC tumor suppressor genes in colon carcinogenesis. So far, it has been reported that PTEN inhibits nuclear translocation of  $\beta$ -catenin, a role usually reserved for wild-type APC (Persad et al., 2001). An interaction between these two suppressor genes warrants further investigation. It would also be interesting to determine if PTEN mutations are affected by microsatellite instability as this data suggests that PTEN is regulated differently in RER+ cells. Recently, a small study of MIN colorectal tumors showed an 18% PTEN mutation rate (Guanti et al., 2000). Perhaps PTEN and APC work in conjunction to determine if a tumor has a MIN or CIN phenotype. From our data, PTEN also appears to be a K-ras-modulated tumor suppressor gene. As PTEN is implicated in colorectal carcinomas in conjunction with APC and K-ras, loss of this tumor suppressor gene may be a new addition to the Vogelstein model of colon tumorigenesis (Fearon and Vogelstein, 1990).

COX-2 regulation occurs through a complex network of signaling pathways and is affected by numerous genetic alterations (Figure 10). It is becoming clear that COX-2

regulation differs depending on the species studied. In rat intestinal epithelial cells, H-ras activation led to a modest transcriptional induction (Sheng et al., 1998), while we observed that K-ras activation in human Caco-2 cells suppressed COX-2 transcription. This difference in COX-2 transcriptional regulation could be of result of sequence differences between the H- and K-ras genes or uncharacterized dissimilarities between the rat and human cell lines. Thus, it is not appropriate to generalize about COX-2 regulation when multiple studies are comparing different species, different tissues (small intestinal versus colonic epithelium) and different genetic mutations. Another disparity found in comparing COX-2 regulation in various cell types is that the signaling pathways are often differentially regulated. For example, the activation of MEK/ERK is essential for induction of K-ras-mediated induction of COX-2 promoter activity and mRNA stability in rat intestinal epithelial cells (Sheng et al., 2001) but MEK/ERK plays only a minor role in the induction of COX-2 protein in human K-ras activated Caco-2 cells (data not shown). Thus, even the same point mutation in K-ras will yield distinct consequences in rat cells versus human cells.

In addition, COX-2 expression levels differ among human colorectal carcinoma cell lines. HT-29 cells express less COX-2 protein than Caco-2 cells, although both cell lines have a wild-type K-ras and a mutant APC. This variance in human cell lines suggests that a human tumor may contain a wide-range of COX-2 expression levels, and thus, COX-2 selective inhibitors may not act as anti-tumor agents in all patients or in all stages of colon tumorigenesis. Thus, it is essential to elucidate the mechanisms of COX-2

**regulation in cells with a variety of common genetic mutations and COX-2 expression levels to better understand and then target COX-2 overexpression in human cancers.**



**Figure 10. Role of genetic risk factors on COX-2 regulation.** K-ras and APC regulate COX-2 expression posttranscriptionally. PKC and K-ras mediate COX-2 expression in Caco-2 cells but not in HT-29 cells. K-ras induces PTEN expression, which negatively regulates the PI-3K signaling pathway. Wild-type APC suppresses PI-3K-dependent signaling of COX-2 protein. An activated K-ras can block the downregulation of COX-2 protein induced by wild-type APC.

## **CHAPTER 4:**

### **SIGNALING OF COX-2 BY THE INTESTINAL LUMINAL BILE ACIDS**

#### **Introduction**

Colon cancer risk is a complex combination of genetic mutations and behavioral factors. The K-ras oncogene is found to be activated in approximately 47% of human colon cancers (Vogelstein et al., 1988), and APC protein is truncated in 90% (Iwamoto et al., 2000). The APC mutation is thought to be an initiating mutation for colon cancer as the majority of both sporadic and familial types of colon cancers harbor these mutations. K-ras mutations are found later in the progression of colon carcinogenesis and lead to larger polyps that are more likely to advance to carcinomas than their normal K-ras counterparts (personal communication, Elena Martinez, University of Arizona)

Dietary factors also play an important role in colon carcinogenesis. Diets high in fat and low in fiber flood the GI tract with copious amounts of bile acids which are then metabolized by the bacteria residing in the gut into secondary bile acids. Secondary bile acids, like deoxycholic acid (DCA), are known colon tumor promoters (Reddy and Wynder, 1973; Narisawa et al., 1974; Reddy et al., 1977; Miwa et al., 1994).

Secondary bile acids are known to transcriptionally signal genes like COX-2. COX-2 is found to be upregulated in approximately 85% of human colon carcinomas compared to normal colonic mucosa (Eberhart et al., 1994) and its expression can be modulated by K-ras and APC mutations (Taylor et al., 2000) (Hsi et al., 1999). K-ras

prevents COX-2 mRNA degradation by stabilization of the ARE elements in the 3'-UTR of the transcript which leads to an increase in protein levels (Sheng et al., 2001). APC, on the other hand, is thought to act by a translational mechanism as wild-type APC does not effect COX-2 mRNA levels but does downregulate COX-2 protein levels compared to cells expressing a mutant APC.

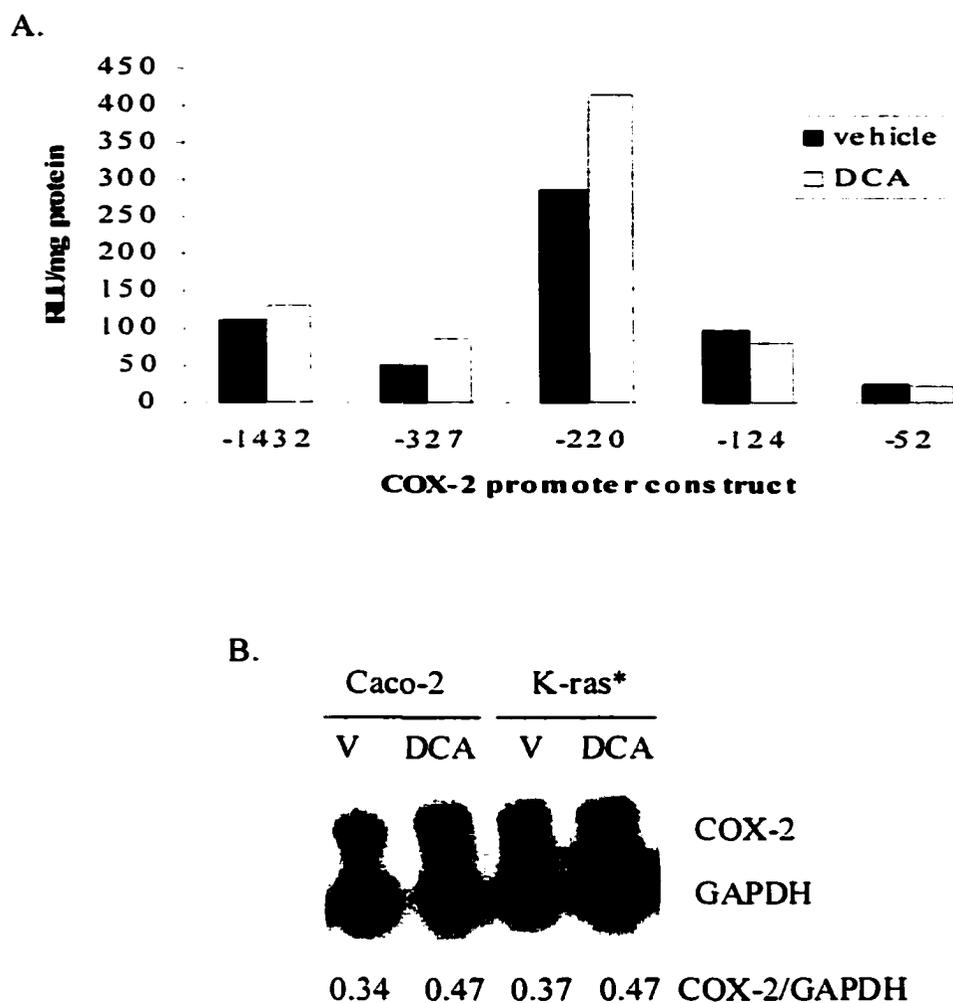
DCA has been reported to upregulate COX-2 at the transcriptional level in both esophageal and gastrointestinal carcinoma cell lines (Zhang et al., 1998; Glinghammar and Rafter, 2001). This regulation seems to work through the cAMP response element on the COX-2 promoter and involves AP-1 activation (Zhang et al., 1998). DCA mediated AP-1 activation has been shown to require PKC and MAPK signaling for downstream gene expression (Qiao et al., 2000).

This study investigated the effects of DCA on COX-2 expression when the K-ras and APC genes are either normal or mutated. These two genes were manipulated in two colon adenocarcinoma-derived cell lines, Caco-2 and HT-29. Both Caco-2 and HT-29 cells have a mutant APC and a normal K-ras. To investigate the role of K-ras activation in DCA-induced COX-2 expression, Caco-2 and K-ras activated Caco-2 cells were studied. To investigate the role of a full-length APC in DCA-induced COX-2, the HT29-APC and HT29- $\beta$ Gal cell model system was utilized. This study showed that both activated K-ras and wild type APC can influence DCA-mediated COX-2 expression. In addition, DCA was able to modulate COX-2 at the posttranslational level by stabilizing the protein.

## **Results**

### ***DCA effects on COX-2 transcription***

Multiple studies have shown that DCA induces COX-2 transcription in epithelial cells - both esophageal and colon cancer cell lines (Zhang et al., 1998; Glinghammar and Rafter, 2001). However, when the effect of DCA on 1400 base pairs of the COX-2 promoter region was tested in our Caco-2 cell model, the increase in promoter activity was minimal (Figure 11A). A similarly small induction, 1.2%, was seen in steady state RNA (Figure 11B). Overall, DCA does not appear to transcriptionally regulate COX-2 in the Caco-2 or K-ras activated Caco-2 cell lines. The difference in transcriptional regulation could be a cell line-specific effect. The colon cells cancer tested in the other study were HCT-116 cells, a COX-2 null line. HCT-116 cells might not have been the best choice for that study as it was an artificial induction of a gene that is not normally expressed in that cell line. However, when we repeated that experiment with HCT-116 cells, a 2-fold induction of promoter activity of the -1432/+59 construct was observed (data not shown).



**Figure 11. Effects of deoxycholic acid on COX-2 transcription.** A. Caco-2 cells were transiently transfected with the COX-2 promoter-reporter constructs -1432/+59, -327/+59, -220/+59, -124/+59, or -52/+59. The cells were then treated with 250  $\mu$ M DCA for 6 h and luciferase activity determined. Luciferase values were normalized to protein concentration. B. Caco-2 and K-ras activated Caco-2 cells were treated with 250  $\mu$ M DCA for 6 h and then total RNA was harvested for COX-2 northern blot analysis. GAPDH served as a loading control.

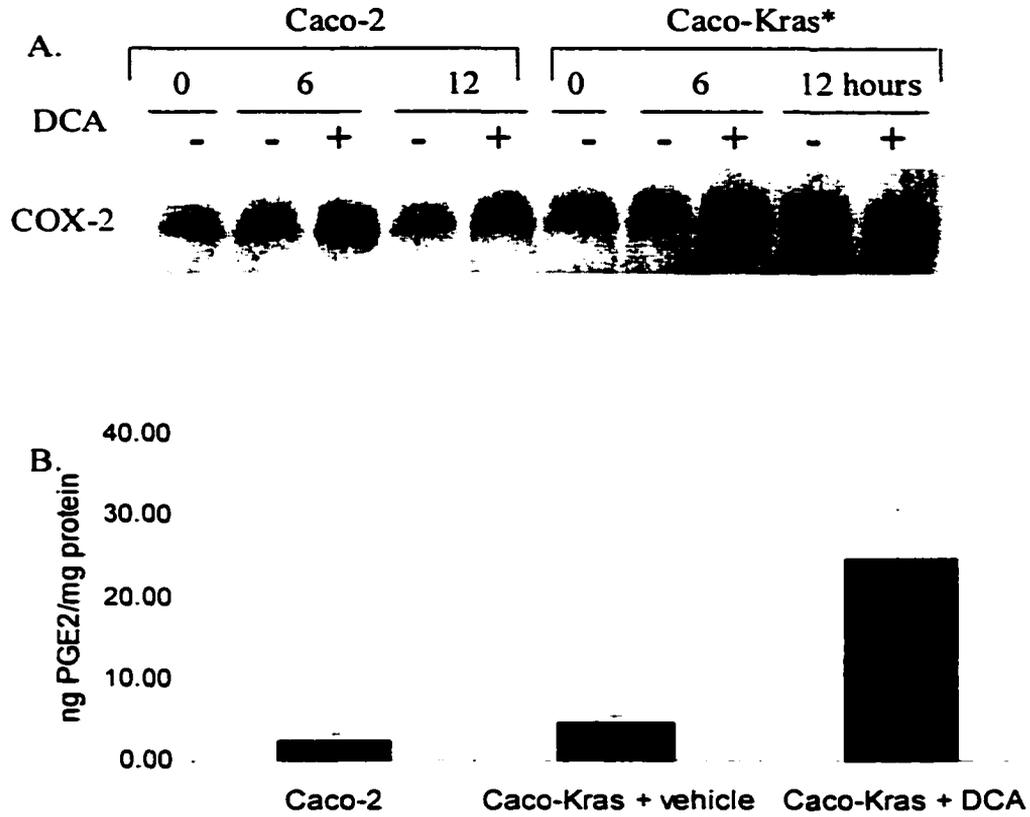
### ***DCA effects on COX-2 protein stability***

The greatest effect of DCA on COX-2 expression was on protein expression (Figure 12A) in the Caco-2 parental cell line. Although DCA induced COX-2 protein to some extent in the K-ras activated line, this effect was not as great or as sustained as that seen with the parental line. This was somewhat surprising since K-ras point mutation was shown to enhance DCA effects in the AOM-treated rat model (Narahara et al., 2000). The DCA-mediated increase in protein expression translated to an increase in prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) levels (Figure 12B).

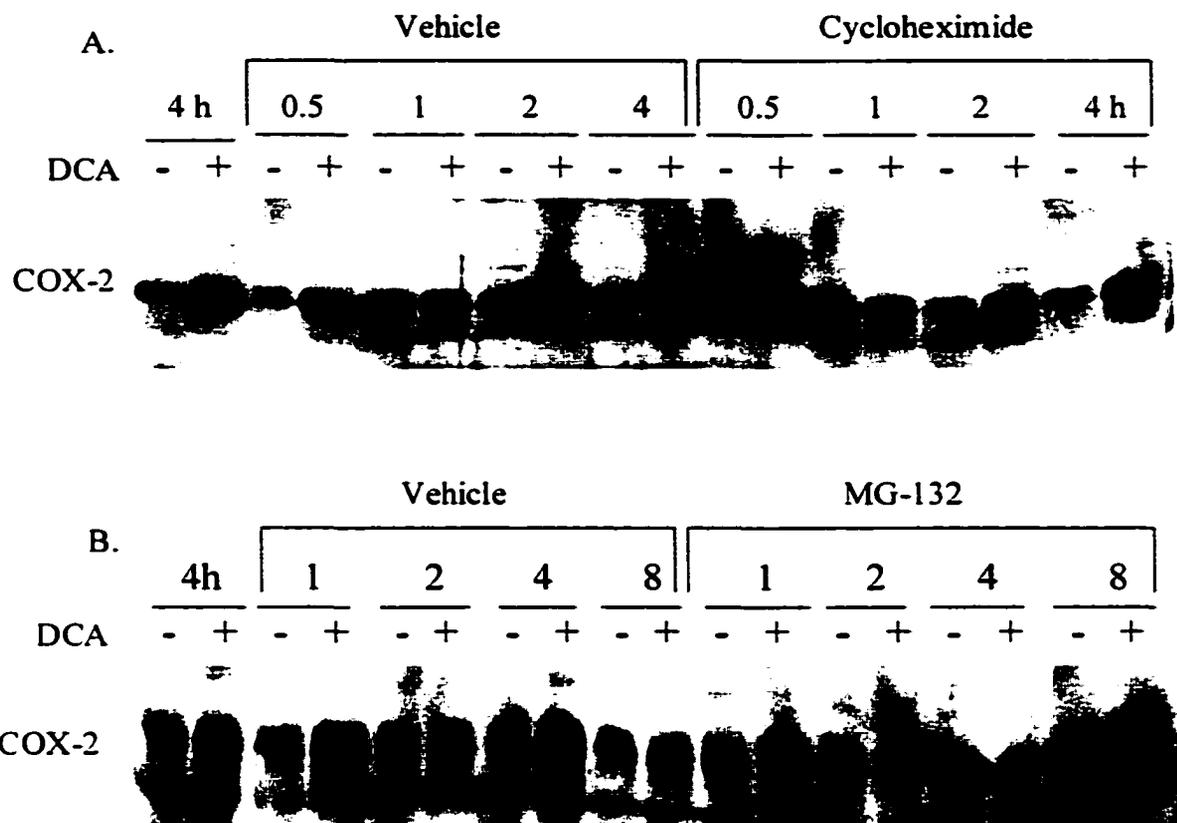
Since the greatest effect of DCA on COX-2 was seen at the level of protein, it seemed to be a natural progression to check the effects of DCA on COX-2 protein stability. This was accomplished by pretreating cells for 4 hours with 250 μM DCA to obtain the COX-2 induction and then treating the cells with the translational inhibitor, cycloheximide. Over time, COX-2 protein decreased in the cycloheximide-treated cells, as would be expected when translation is blocked. However, the protein levels did not decrease in the presence of DCA (Figure 13A). Thus, DCA appeared to stabilize COX-2 protein.

A similar result occurred in the presence of the proteasome inhibitor, MG-132. COX-2 protein is degraded by the 26S proteasome and treatment with MG-132 will prevent this degradation, so that COX-2 protein is allowed to build up in the cell, with an increase in protein that is measurable by western blot (Shao et al., 2000). Again, cells were stimulated with DCA to begin the COX-2 induction, and then cells were treated with MG-132 to inhibit protein degradation. Over the 8 hour period, the COX-2 protein

increased in the vehicle (for DCA) and MG-132 treated cells (Figure 13B). To an equal extent, COX-2 protein increased in the cells treated with DCA and MG-132. It does not appear that the increase in the DCA + MG-132-treated cells is due to a synergetic effect of DCA and MG-132. It can be inferred from this data that DCA stabilizes COX-2 protein to the same extent as does a proteasome inhibitor.



**Figure 12. DCA induces COX-2 protein expression and enzyme activity.** A. Caco-2 and K-ras activated Caco-2 cells were treated with 250  $\mu$ M DCA for 0, 6, 12 h and harvested for COX-2 western blotting. B. K-ras activated Caco-2 cells were treated with 250  $\mu$ M DCA or H<sub>2</sub>O vehicle for 6 h and then COX enzyme activity was stimulated with 15  $\mu$ M arachidonic for 1 h in the presence of drug. The media was harvested to determine extracellular PGE<sub>2</sub> amounts. PGE<sub>2</sub> production was normalized to protein concentration.

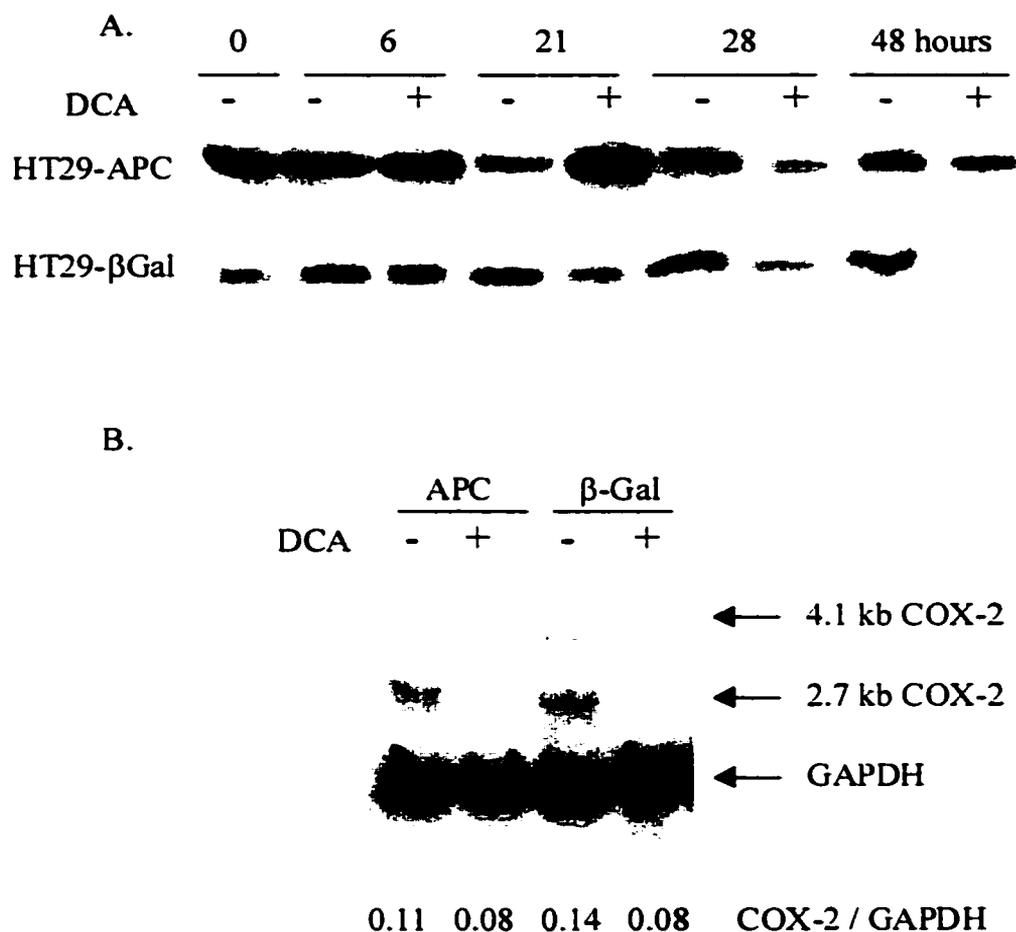


**Figure 13. DCA promotes COX-2 protein stability.** A. Caco-2 cells were pretreated with 250  $\mu$ M DCA for 4 h to induce COX-2 protein before addition of 50  $\mu$ M cycloheximide or H<sub>2</sub>O vehicle. Cells were lysed after 0.5, 1, 2 or 4 h of treatment and analyzed for COX-2 protein expression. A representative of three experiments is shown here. B. Caco-2 cells were pretreated with 250  $\mu$ M DCA for 4 h to obtain the induction of COX-2 protein before addition of 10  $\mu$ M of the proteasome inhibitor, MG-132. A representative of two experiments is shown.

### ***Involvement of APC in DCA-mediated COX-2 Expression***

Similar experiments were performed in the HT-29 cell model with an inducible wild-type APC to determine the role, if any, APC played in bile acid-mediated COX-2 expression. HT29-APC and HT29- $\beta$ Gal cells were pretreated with ZnCl<sub>2</sub> for 6 hours to fully induce wild-type APC before subsequent treatment with DCA. DCA induced COX-2 protein in the HT29-APC cells by 21 h and then the induction quickly tapered off as it was not sustained through the 28 hour timepoint (Figure 14A). Interestingly, DCA was not able to induce COX-2 protein in HT29- $\beta$ Gal cells at any of the timepoints tested.

DCA induced COX-2 in the HT29-APC cell model only at the protein level, since steady state levels are actually reduced after treatment with DCA (Figure 14B). It would be interesting to determine if DCA is stabilizing COX-2 protein in this cell line with the cycloheximide and MG-132 experiments.



**Figure 14. Involvement of APC in DCA-mediated COX-2 Expression.** A. HT29-APC and HT29-βGal cells were pretreated with 300 μM ZnCl<sub>2</sub> for 6 h and then treated with 250 μM DCA or vehicle. Cells were harvested at various timepoints for COX-2 western blotting. B. COX-2 northern blot analysis of HT29-APC and HT29-βGal cells treated with 250 μM DCA in the presence of ZnCl<sub>2</sub>. COX-2 mRNA levels were normalized to GAPDH mRNA levels to control for loading differences.

## **Summary**

These experiments are the first to implicate bile acids in COX-2 protein stability. Cells treated with cycloheximide to inhibit translation show a decrease in COX-2 protein over time. Cells treated with MG-132 to prevent degradation by the 26S proteasome, show an increase of COX-2 protein over time. In both cases, DCA can induce COX-2 protein, stabilizing it as much as the proteasome inhibitor. This finding is in contrast to one study showing that DCA can potentiate proteosomal degradation of p53 (Qiao et al., 2001). It is interesting that DCA can both stabilize oncogenes like COX-2 and destabilize tumor suppressor genes like p53, both of which will result in potentiation of the tumorigenesis process.

As mentioned in Chapter 3, Figures 5 and 6, the PKC signaling mechanism is altered in some way in the HT-29 cells. TPA or an activated K-ras were unable to induce COX-2 in these cells as they did in the Caco-2 cell model. This PKC-signaling deficiency may be coming into play here as well. The HT29-βGal cells still have a mutant APC and could be expected to behave just as their parent cell line, HT-29. Since it has already been proven that they are insensitive to TPA stimulation of COX-2, and it is established that DCA primarily works through PKC signaling (Craven et al., 1987; Huang et al., 1992), then it is not surprising that these cells are insensitive to DCA stimulation of COX-2.

These data also show that a wild-type APC can work to reverse the repression on bile acid-mediated induction of COX-2 in HT-29 cells. When a wild-type APC is induced in the HT29-APC cells, DCA is able to induce COX-2 protein expression. If bile acid-

mediated signaling of COX-2 is dependent on PKC in the the HT-29 cells, then perhaps wild-type APC (in the HT29-APC cells) is restoring PKC signaling in these cells.

Not much is reported in the literature about an interaction between APC and PKC. There are only a few studies suggesting that APC may regulate PKC activity. Min mice, which have a mutant APC, show decreased levels of the  $\alpha$ ,  $\beta 1$ ,  $\zeta$  and  $\mu$  PKC isoforms (Klein et al., 2000). On the other hand, another study shows that PKC activation by TPA causes  $\beta$ -catenin translocation to the nucleus independently of APC status (Baulida et al., 1999). It is still unclear as to how APC may be regulating PKC (or vice versa), yet it is possible that these two genes may be working together to modulate expression of a downstream gene like COX-2.

If the DCA-mediated induction of COX-2 in cells with wild-type APC was solely mediated by PKC, then one would expect APC to surmount the suppression of the K-ras activation of COX-2 in HT-29 cells, which it does. As seen in chapter 3, a wild-type APC permits K-ras activation to induce COX-2 in the HT-29 cell model (Figure 8). It is still unknown why K-ras activation of COX-2 is dependent upon a wild-type APC in the HT-29 cells but does not need a wild-type APC in the Caco-2 cells. This finding leaves open the possibility that the principal mechanism of K-ras-mediated upregulation of COX-2 is through PKC in the HT-29 cells.

**CHAPTER 5:**  
**SULINDAC SULFONE INHIBITS K-RAS DEPENDENT CYCLOOXYGENASE-2**  
**EXPRESSION**

**Introduction**

The cyclooxygenase pathway has been under intense investigation as a target for treatment and prevention of colorectal cancer. Several studies have reported a 40-50% decrease in mortality from colorectal cancer with prolonged use of NSAIDs, which block COX enzyme activity (Kune et al., 1988; Rosenberg et al., 1991; Thun et al., 1991; Pelage et al., 1994). Sulindac, an inhibitor of both COX-1 and COX-2, has been reported to reduce the size and number of colorectal tumors in familial adenomatous polyposis patients (FAP) as well as in animal models of FAP (Giardiello, 1996). Sulindac is metabolized into sulfone and sulfide derivatives. Both derivatives are known to inhibit cell growth by the induction of apoptosis (Piazza et al., 1995). The sulfide metabolite has been shown to have COX enzyme inhibitory activity, while the sulfone metabolite lacks this activity (Duggan et al., 1977; Shen and Winter, 1997). Recent studies have shown that sulfone can prevent tumor formation in rodent models of chemical carcinogenesis without inhibiting COX enzyme activity or lowering prostaglandin levels (Piazza et al., 1995; Thompson et al., 1997). The sulfone metabolite, which is now referred to as exisulind (Aptosyn™) is currently being developed for the treatment of FAP patients as well as other cancer chemopreventive and therapeutic indications.

The first evidence linking an activated ras oncogene to upregulation of COX-2 was one in which an activated H-ras gene was transfected into rat intestinal epithelial cells (Sheng et al., 1997). Treatment with the COX-2 selective inhibitor, SC58125, suppressed growth in these cells and induced apoptosis. An activated H-ras transfected into rat-1 fibroblasts caused an increase in transcription and in the half-life of COX-2 message, which appeared to be modulated by the MAPK pathway (Sheng et al., 1998). Selective inhibition of MEK by PD98059 caused a delay in COX-2 induction at both mRNA and protein levels. This suggests that the MAPK signaling cascade may mediate COX-2 expression.

Sulindac sulfide has previously been shown to bind ras to inhibit the recruitment of raf to the plasma membrane and thus directly inhibit ras signaling (Hermann et al., 1998). In addition, sulindac sulfone and certain NSAIDs (i.e., piroxicam and sulindac) have been previously shown to have a proportionately greater inhibitory effect on carcinomas harboring mutations in either K- or H-ras (Singh et al., 1994; Thompson et al., 1997). In order to determine if sulindac sulfone can affect ras-dependent signaling, we investigated the effect of this drug on K-ras-dependent expression of COX-2 in a human colon cancer derived cell line.

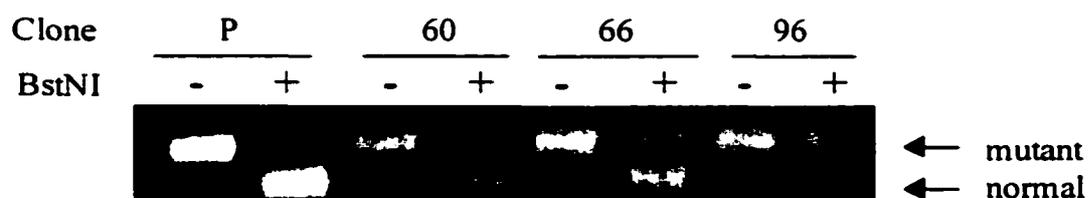
## **Results**

### ***Characterization of K-ras activated clones***

Caco-2 cells were used for stable transfection of p21<sup>Val12</sup> K-ras because they express normal K-ras (Singh et al., 1994). In order to better elucidate K-ras overexpression, a PCR-RFLP technique was used to determine if the transfectants

overexpressed the normal or mutant alleles. A BstNI restriction enzyme cut site was introduced using specific primers and then the PCR product was cut. The enzyme cuts if the cells are expressing the normal allele but it will not cut if the cells are expressing the mutant allele. The data shows that the clones with the highest expression (60, 66, 96) displayed both normal and mutant alleles, while the parental cell line expressed only normal K-ras (Figure 15). This shows that the transfected cells do indeed express the mutant K-ras gene but does not provide information regarding the level of the normal or the mutant allele being expressed.

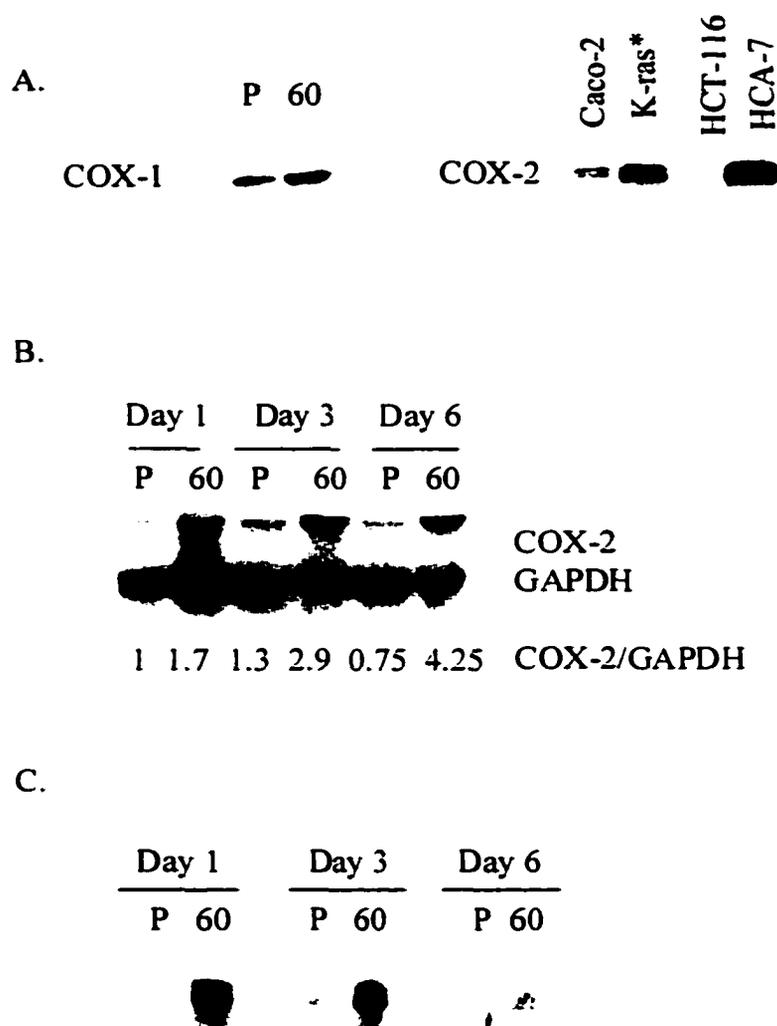
Overexpression of K-ras protein in the K-ras activated clones (6, 26, 60, 66, 96) was verified by western blotting (Lawson et al., 2000). Data generated by another lab member suggests that both the normal and mutant alleles are being overexpressed simultaneously in the transfectants, as there is no difference in growth rate.



**Figure 15. The characterization of the mutational status of K-ras activated Caco-2 clones.** Caco-2 parental cells (P) and K-ras activated clones (60, 66, 96) were tested for normal and mutant alleles of K-ras with the use of a PCR-RFLP technique. PCR products were digested with BstNI; mutation at codon 12 prevents BstNI cleavage.

***Cyclooxygenase Expression in K-ras activated Caco-2 cells***

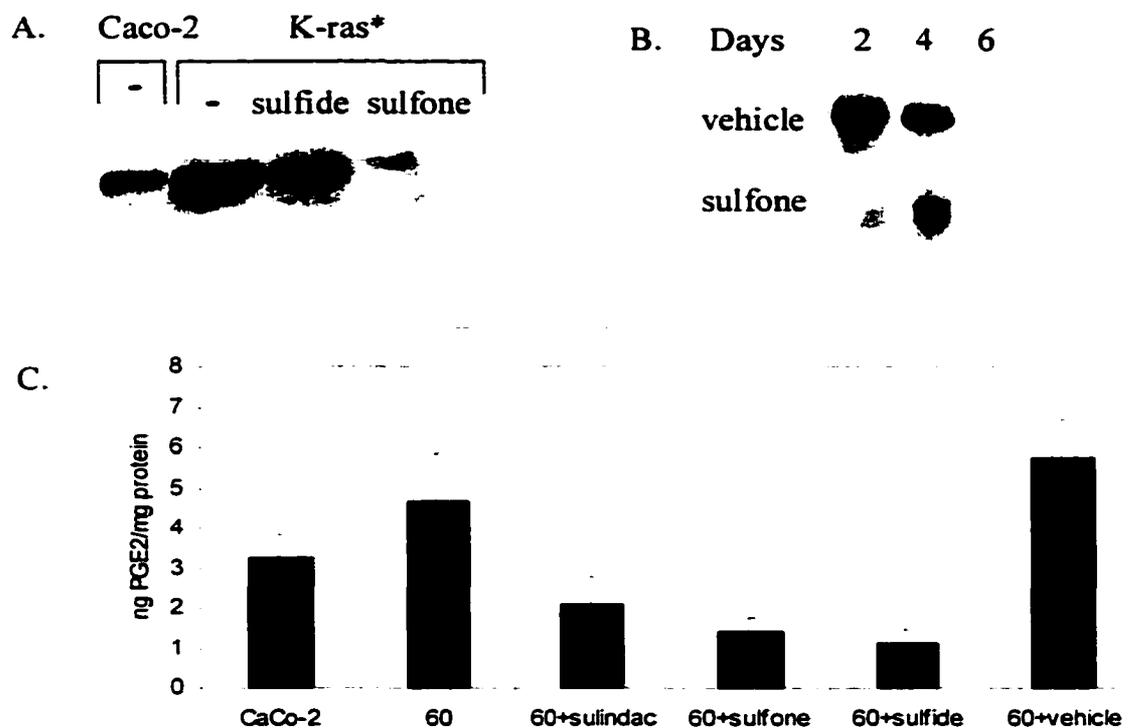
COX-2 protein and RNA levels were measured in the K-ras activated clones and the parental Caco-2 cell line. The level of COX-2 protein was increased in the K-ras activated cells relative to the parental cell line but no difference was observed in COX-1 protein (Figure 16A). There was a similar increase in COX-2 mRNA levels in the K-ras activated clones (Figure 16B). This data suggests that an activated K-ras increases the expression of COX-2 mRNA and protein. The increase in expression of COX-2 protein was transient, peaking at 2-3 days in culture and decreasing over time (Figure 16C). This transient effect is irrespective of K-ras status, as would be expected since COX-2 is an inducible rather than constitutive gene.



**Figure 16. Effect of K-ras on COX-2 Expression.** A. Western blot analysis of COX-1 and COX-2 in Caco-2 parental cells (P) and K-ras activated clones (60) after 48 h in culture. HCT-116 cells served as a negative control for COX-2 and HCA-7 cells are a positive control as they constitutively express COX-2. B. Northern blot of COX-2 in Caco-2 and 60 cells at 1, 3, 6 day time points. Amounts of COX-2 mRNA were expressed in a ratio to GAPDH levels, normalized to parentals on day 1. C. Time course of COX-2 protein levels in parental and 60 cells at days 1, 3, 6 in culture.

***Effects of Sulindac and its Sulfide and Sulfone Metabolites on COX-2 Expression, PGE<sub>2</sub> Production and Tumorigenesis***

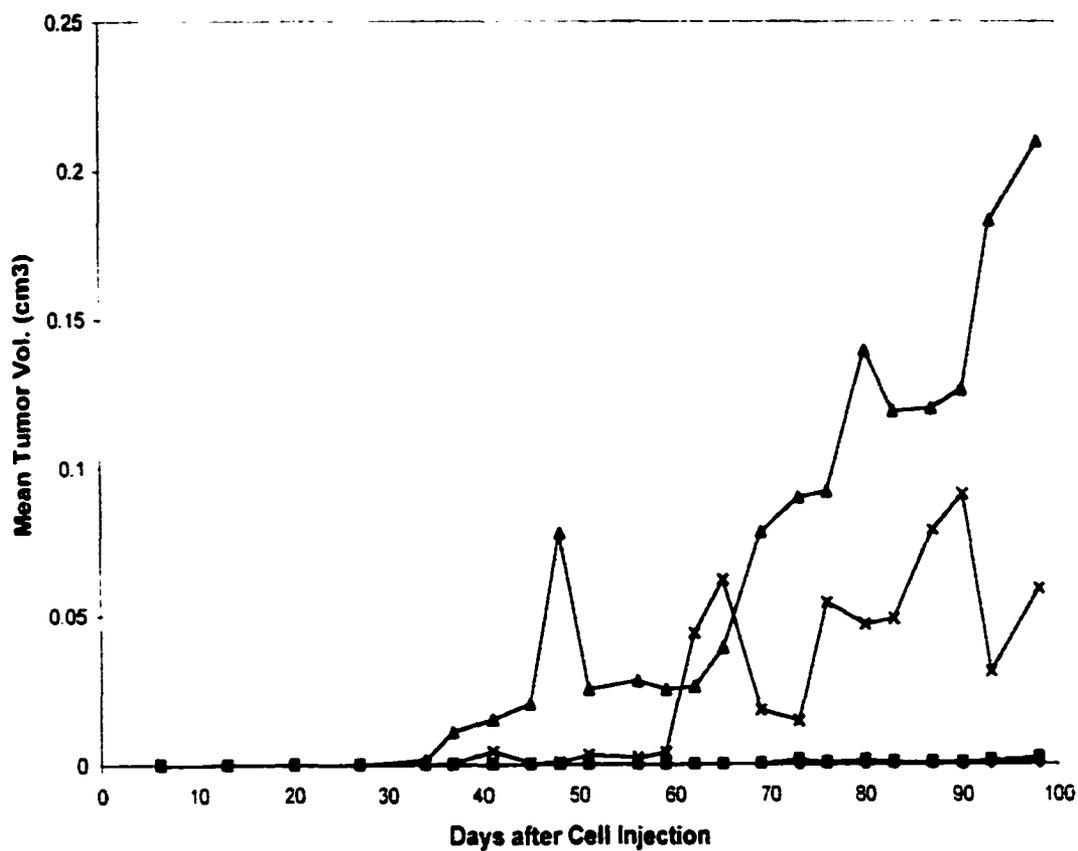
Since K-ras activated Caco-2 cells exhibited an elevation in COX-2 mRNA and protein, we expected to observe an increase in PGE<sub>2</sub> production in K-ras transfected cells, compared to the parental Caco-2 cells. K-ras activated cells showed a 30% increase in PGE<sub>2</sub> synthesis over the parental cells (Figure 17C). PGE<sub>2</sub> production was next measured in cells treated for 24 hours with sulindac and its sulfide and sulfone metabolites at doses previously established to inhibit Caco-2 colony formation by 50%. Treatment with 400 μM sulindac sulfoxide (the parent sulindac compound) significantly reduced PGE<sub>2</sub> production in K-ras activated cells. Sulindac sulfone at 600 μM and sulfide at 120 μM displayed similar inhibitory effects on prostaglandin synthesis (Figure 17C). In addition, sulfone significantly inhibited COX-2 protein (and mRNA, data not shown), while the sulfide at its IC<sub>50</sub> dose did not affect protein levels (Figure 17A,B). This finding suggests that the sulfone can indirectly affect prostaglandin synthesis by affecting COX-2 expression. By contrast, the sulfide inhibits COX activity but does not affect expression. This is the first demonstration that sulindac sulfone can exert cyclooxygenase-dependent effects on cell behaviors, specifically by suppressing K-ras dependent COX-2 expression.



**Figure 17. The effects of sulindac sulfone on COX-2 expression and PGE<sub>2</sub> production.** A. Western blot of COX-2 expression in Caco-2 and K-ras transfectants. K-ras transfectants were treated for 24 h with 120  $\mu$ M sulfide or 600  $\mu$ M sulfone. B. COX-2 protein levels in the presence of 600  $\mu$ M sulindac sulfone on days 2, 4, 6. C. Extracellular PGE<sub>2</sub> levels in Caco-2, K-ras transfectants, and K-ras transfectants treated with 400  $\mu$ M sulindac, 600  $\mu$ M sulfone, 120  $\mu$ M sulfide, or DMSO vehicle for 24 h. *P* for vehicle-treated cells compared with sulfone- or sulfide-treated cells are *P* = 0.02 and *P* = 0.003, respectively.

***Effects of sulindac on tumor formation of Caco-2 and K-ras transfected cells injected into SCID mice***

In order to determine the physiological relevance of sulindac's inhibition of COX-2 RNA and protein, SCID mice were injected with cancer cells and fed a diet containing sulindac. Sulindac inhibited tumor formation in SCID mice injected with K-ras activated Caco-2 cells (Figure 18). Caco-2 cells and the K-ras activated transfectants showed no difference in growth characteristics in vitro (Ref: Kathryn R. Lawson's dissertation, 1998) but displayed differences in tumor formation when injected in immune-compromised mice. K-ras activation caused an increase in the rate of tumor formation over the nontransfected cell line, and sulindac greatly inhibited this tumorigenesis. Measurable tumors became apparent in untreated mice within 30 days, while sulindac-treated mice did not present with tumors until day 60. By day 100, sulindac had prevented tumor growth by 60% over the control group.



**Figure 18. Effects of sulindac on tumor formation on Caco-2 and K-ras transfectants injected into SCID mice.**  $1 \times 10^6$  cells in 0.2 ml PBS were injected at 4 sites subdermally, and 167 parts/million sulindac were administered in the diet. Tumor growth was measured twice a week. Caco-2 (■), Caco-2 + sulindac (●), 60 (▲), 60 + sulindac (✕). *P*'s were calculated at 100 days using ANOVA. *P* was <0.001 for Caco-2 and 60 cells, *P* = 0.01 for 60 and 60 + sulindac.

## Summary

The data presented here indicate that sulindac and its sulfide and sulfone metabolites inhibit tumorigenesis in colon derived cells by at least two distinct mechanisms. One mechanism involves the suppression of prostaglandin synthesis by COX-1 and/or COX-2 enzyme activity (Shen and Winter, 1997). The second mechanism, shown here for the sulfone metabolite, involves inhibition of K-ras dependent signaling of genes affecting tumorigenesis. In our Caco-2 model, one of these genes includes COX-2.

IC<sub>50</sub> concentrations of both the sulfide (120 μM) and the sulfone (600 μM) metabolites that inhibit colony formation were used in these experiments. The increased potency of sulindac sulfide may reflect the ability of this metabolite to suppress COX enzyme activity and prostaglandin synthesis, while a higher concentration is required to accomplish these same endpoints by suppressing K-ras dependent signaling. This suggests that higher concentrations of sulindac sulfide may also inhibit COX-2 protein expression. Hermann et. al. (Hermann et al., 1998) have reported that sulindac sulfide can bind to ras in cell free studies. Future studies will address whether the sulfide or sulfone directly bind to ras in human colon cancer cells.

The reduction in PGE<sub>2</sub> in the sulindac-treated K-ras activated Caco-2 cells may be due to its effects on COX-1 or COX-2 or both. The distinction cannot be made by measuring prostaglandin levels. Yet we hypothesize that it is working by a selective COX-2 inhibitory mechanism in cells with mutant K-ras because we observe no change

in COX-1 protein levels in the K-ras activated cells (Figure 16A). COX-1 contributes to prostaglandin synthesis overall, but it may not be playing a significant role in our model.

Modulation of K-ras-dependent gene expression by sulindac sulfone does not seem to be limited to COX-2. We have observed an increase in promoter activity and enzyme activity in the polyamine catabolic enzyme, spermidine/spermine N<sup>1</sup>-acetyltransferase (SSAT) when K-ras activated Caco-2 cells are treated with sulfone (N.A. Ignatenko et. al., manuscript in preparation). This suggests that sulfone is acting to modulate the K-ras dependent signaling of a variety of genes influencing multiple cellular processes, including cellular proliferation and survival. Sulindac acts by both COX-dependent and COX-independent pathways. COX-independent mechanisms include the suppression of COX enzyme expression that interferes with COX-2-mediated signaling of other genes (Hanif et al., 1996) as well as inducing apoptosis by activating protein kinase G (Thompson et al., 2000).

The data presented here provide additional evidence for the role of COX-2 expression in colon carcinogenesis, where up to 50% of large adenomas and adenocarcinomas contain activating mutations in K-ras (Hanif et al., 1996). We showed that an activated K-ras oncogene leads to an upregulation of COX-2 expression in human adenocarcinoma cells. Previous work has shown that activated H-ras leads to upregulation of COX-2 in rodent and human colon-derived cells (Delage et al., 1993; Sheng et al., 1998). However, H-ras is not expressed in human colonic epithelium, while K-ras mutations occur with high frequency in colorectal cancers (~50%) and are found in nearly 100% of pancreatic cancers (Bos et al., 1987; Forrester et al., 1987; Almoguera et

al., 1998). K-ras has been shown to be the only ras family member to be non-essential for development in mice (Johnson et al., 1997). Thus, it is possible that, in humans, K-ras may have functions unique from H-ras, as has been shown for mice (Johnson et al., 1997).

This study describes a novel COX-dependent effect of the NSAID sulindac, namely the inhibition of K-ras dependent expression of COX-2 protein by the sulfone metabolite of this drug. These data, however, support the conclusion that NSAIDs, such as sulindac, also act by non-COX-dependent mechanisms which affect signaling pathways controlling the expression of genes which regulate cell behaviors, including tumorigenesis.

**CHAPTER 6:**  
**POLYAMINES MODULATE COX-2 EXPRESSION THROUGH MECHANISMS**  
**INVOLVING EIF-5A AND POLYAMINE TRANSPORT**

**Introduction**

Polyamines are considered luminal risk factors for colon cancer as they are increased after the ingestion of meat and heavily processed foods like cheeses. Multiple genetic and luminal risk factors for colon cancer have been reported to regulate the expression of COX-2, a protein commonly upregulated in neoplastic colon tissue compared to nearby normal mucosa. There is nothing reported in the literature about an interaction between polyamines and COX-2 expression, even with the existence of an ongoing clinical trial of the combination of DFMO, an inhibitor of ODC activity and sulindac, an inhibitor of COX activity. There are, however, multiple pieces of evidence that suggest that such an interaction may be plausible.

Polyamines are strong mediators of transcription and translation as they can bind to nucleic acids to facilitate transcription factor binding and secondary structure, just to name a few of their functions. Several modifiers of COX-2 expression have been shown to mediate COX-2 RNA and protein expression. Genetic risk factors for colon cancer like K-ras and APC both work posttranscriptionally to mediate COX-2 expression in human colon cancer cells (Hsi et al., 1999; Sheng et al., 2000; Taylor et al., 2000). Our lab has shown that mutations in the K-ras oncogene and the APC tumor suppressor gene

influence polyamine synthesis and catabolism. K-ras activation induces ODC mRNA steady state levels (NA Ignatenko, personal communication) and also inhibits SSAT activity to further increase intracellular polyamine pools (NA Ignatenko, manuscript in preparation). Wild-type APC suppresses ODC activity to prevent the build up of polyamines to toxic levels (Fultz and Gerner, May 2002). Luminal risk factors like bile acids mediate COX-2 both transcriptionally (Glinghammar and Rafter, 2001) and posttranscriptionally as seen in Chapter 5 of this dissertation. Bile acids also activate ODC, possibly through generation of nitric oxide (Takano et al., 1984). As polyamines are another luminal risk factor for colon cancer, it is logical that they could also mediate COX-2 expression to potentiate colon tumorigenesis.

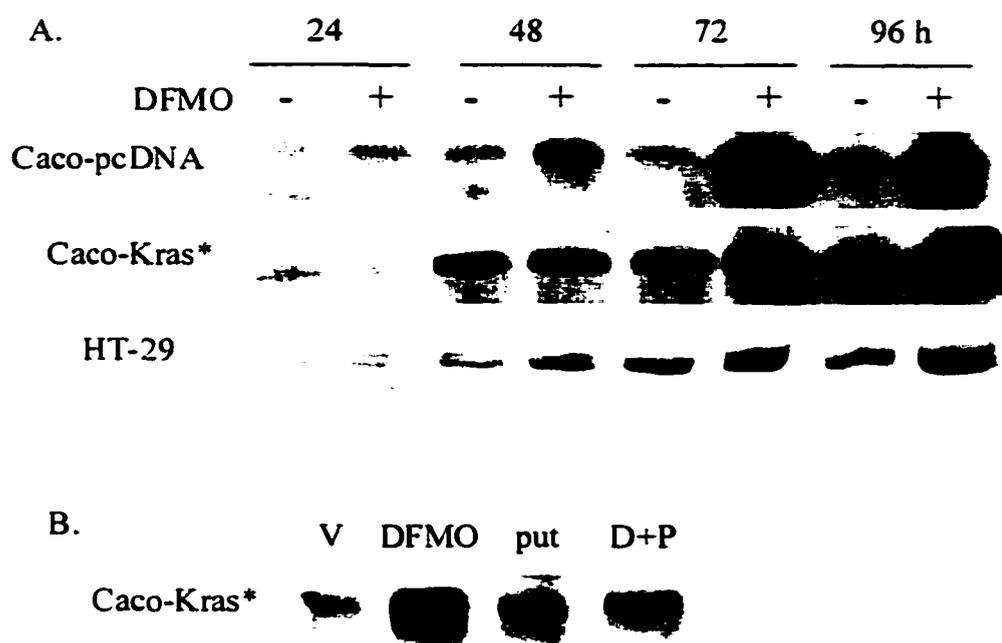
This study set out to determine the role, if any, polyamines were playing in COX-2 regulation. This study also planned to investigate the molecular mechanisms by which combination chemoprevention with sulindac and the ODC inhibitor, DL- $\alpha$ -difluoromethylornithine (DFMO) may be effective. The data show that polyamine depletion by DFMO results in an induction of COX-2 protein levels and that the polyamine-mediated eIF-5A protein plays a role in the RNA processing of the COX-2 message. HCA-7 cells, which constitutively express COX-2, have a polyamine transport defect that may result in this overexpression of COX-2.

## **Results**

### ***Polyamine Depletion Induces COX-2 protein***

In order to determine the effects of polyamines on COX-2 expression, cells were first treated with DFMO, an inhibitor of ornithine decarboxylase (ODC) to inhibit

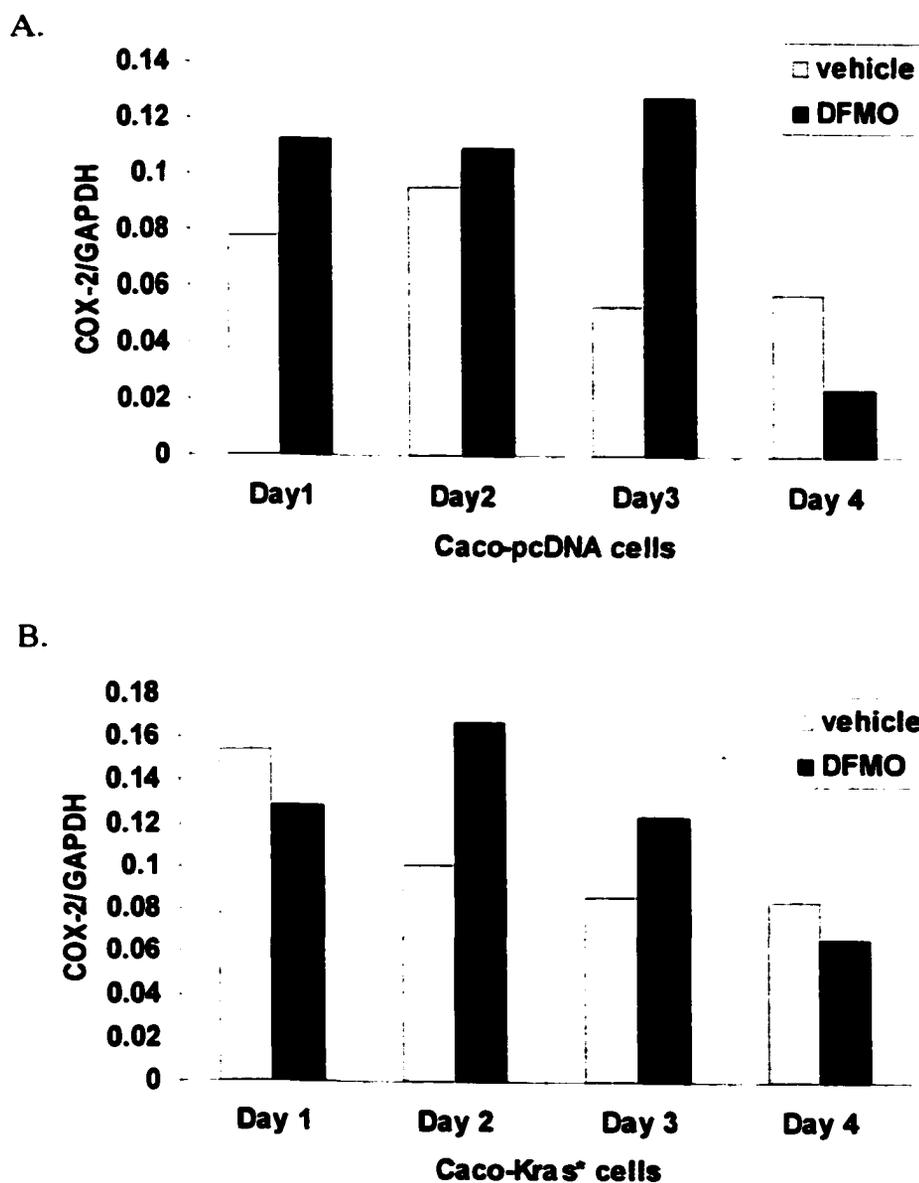
polyamine synthesis. COX-2 expression was measured at both the transcriptional and translational level. In a time-dependent manner, depletion of polyamines by DFMO greatly induced COX-2 protein expression. This phenomena was seen in three colon cancer cell lines expressing various levels of COX-2: Caco-pcDNA (vector only for K-ras), K-ras activated Caco-2 cells, and HT-29 cells. The induction began by 48 h of DFMO treatment in the Caco-2 and HT-29 cells but was delayed until 72 h in the K-ras activated Caco-2 cells (Figure 19A). This induction of COX-2 protein was determined to be polyamine dependent as it could be rescued by addition of putrescine (Figure 19B).



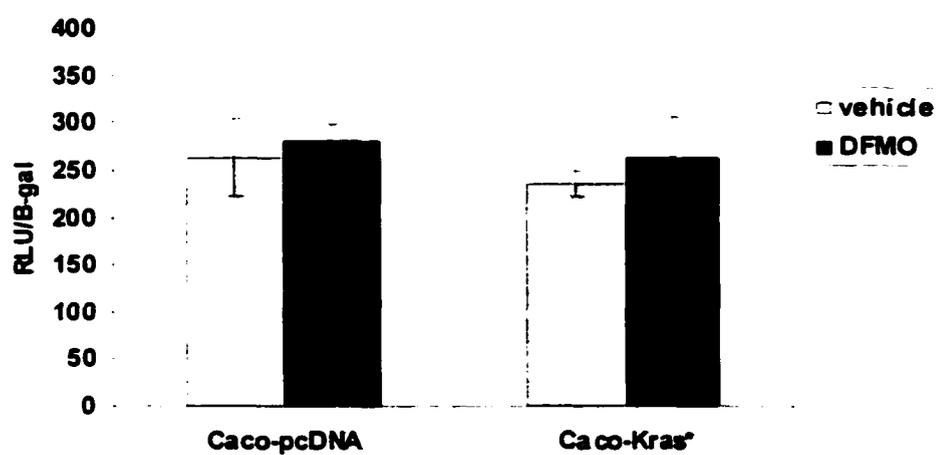
**Figure 19. Polyamine depletion increases COX-2 protein expression.** A. Caco-pcDNA, Caco-Kras and HT-29 cells were treated with 5 mM DFMO or H<sub>2</sub>O vehicle over 4 days and harvested every 24 h for COX-2 western blots. B. Western blot analysis of Caco-Kras cells treated with vehicle (V), 5 mM DFMO, 1 mM putrescine (put) or both DFMO and putrescine (D+P). DFMO treatment preceded putrescine addition by 72 h and putrescine treatment lasted for another 24 h.

The next question asked was if polyamine depletion was acting at the transcriptional or translational level to modulate COX-2 protein. The first experiment performed to answer this question was a COX-2 northern with cells treated with DFMO for over the same four-day time period. DFMO treatment caused a modest induction of COX-2 mRNA but not to the extent as seen with protein expression (Figure 20). DFMO induced steady-state RNA levels on day 1 in Caco-pcDNA cells, which remained elevated by day 3 and then dropped off by day 4. In the K-ras cells, the induction began a day later as seen with the protein levels and remained elevated through day 3.

The induction of steady-state mRNA by DFMO treatment is not due to an increase in promoter activity. The largest COX-2 promoter construct (-1432/+59) was transiently transfected into both the Caco-pcDNA and K-ras activated cell lines and the cells were treated with DFMO for 2-3 days to verify that COX-2 protein was being induced. There was no significant difference in COX-2 promoter activity levels in either cell line treated with DFMO (Figure 21). This suggests that DFMO is regulating COX-2 by a posttranscriptional mechanism.



**Figure 20. Effects of DFMO on COX-2 mRNA steady state levels.** Caco-pcDNA (A) and Caco-Kras (B) cells were treated with 5 mM DFMO for 1-4 days and total RNA was harvested for northern blotting. COX-2 mRNA levels are represented as a ratio to GAPDH mRNA levels to control for loading.



**Figure 21. DFMO does not affect COX-2 promoter activity.** Caco-pcDNA and Caco-Kras cells were transiently transfected with the -1432/+59 COX-2 promoter-reporter construct and then treated with 5 mM DFMO for 2 days. Luciferase activity was determined and normalized to βGal activity and protein concentration.

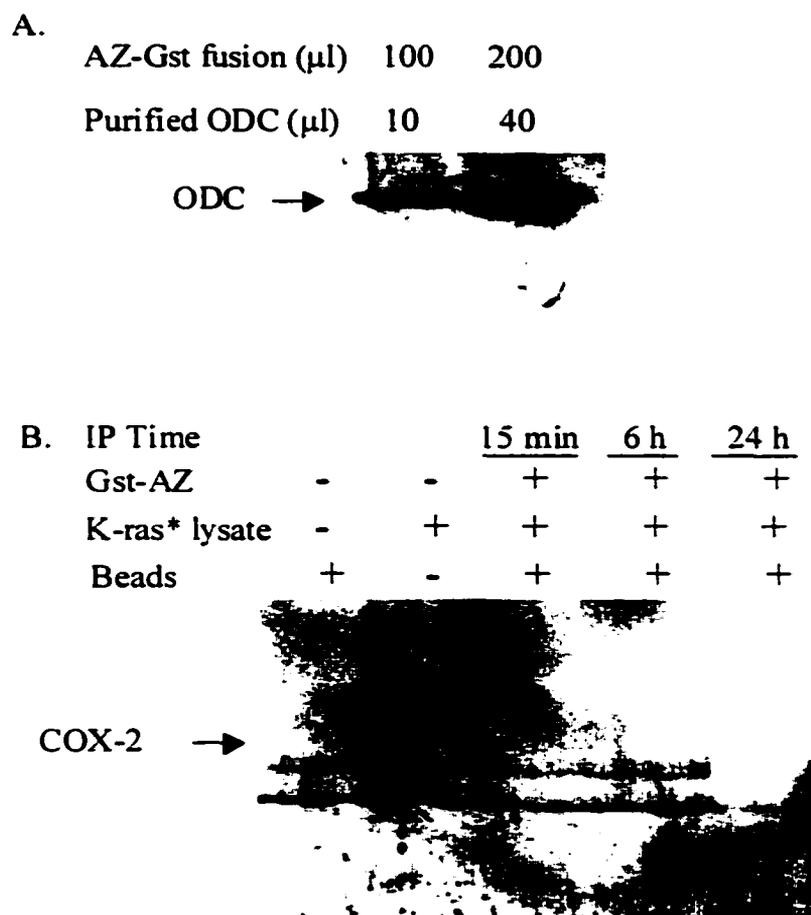
### ***Lack of Polyamine-Mediated COX-2 Protein Stabilization***

Since the greatest effects of DFMO were observed at the protein level, it seemed only natural that polyamines could be acting as the other luminal risk factor studied, the bile acid DCA, and stabilizing COX-2 protein. Pulse-chase experiments were attempted: cells treated with DFMO or vehicle were radioactively labeled, harvested at various time points, and run out on an SDS-PAGE gel for autoradiography. This experiment was attempted several times and proved to be technically challenging and difficult to interpret. However, none of the experiments proved that DFMO was stabilizing COX-2 protein (data not shown).

It was then suggested that antizyme, the negative regulator of ODC, was able to stabilize cell cycle proteins usually degraded by the 26S proteasome {personal communication: Bruce Zetter (Harvard University, Cambridge, MA) to EW Gerner}. Data was put forward showing that antizyme physically bound to cyclin D1 to target it for degradation. Since COX-2, like cyclin D1, is also degraded by the 26S proteasome, it seemed likely that antizyme may bind to COX-2 to influence its degradation. DFMO treatment inhibits ODC activity, thus downregulating antizyme levels. If antizyme was truly binding COX-2 and targeting it for degradation, then DFMO-treated cells would exhibit increases in COX-2 protein because this degradation process would be stunted by decreased levels of antizyme. Since this was the observation made from western blots of DFMO-treated cells (Figure 19), this hypothesis was pursued.

In order to determine if antizyme (AZ) was directly binding to COX-2, a Gst-AZ fusion protein was incubated with colon cancer cell lysate and COX-2 was

immunoprecipitated using GSH-coated beads. Purified ODC protein was used as a positive control for antizyme binding since the main known function of antizyme is to bind to ODC and target it for ubiquitin-independent degradation by the 26S proteasome. As seen in Figure 22, the Gst-AZ protein was able to bind to the ODC protein in a dose-dependent manner. However, antizyme did not bind to COX-2 protein at any of the timepoints tested. These negative results were obtained three times. Thus, the role of polyamines in COX-2 expression is not through AZ-mediated protein stability.



**Figure 22. Antizyme protein does not directly bind to COX-2 protein.** AZ-Gst fusion protein was incubated with Caco-Kras cell lysate or purified ODC protein and glutathione-coated beads. The beads were centrifuged and the pellet run out on an SDS-PAGE gel. The proteins bound to the beads were then transferred to nitrocellulose membrane and probed with ODC (A) or COX-2 (B) antibodies. Abbreviations: AZ, antizyme; ODC, ornithine decarboxylase.

### ***Involvement of eIF-5A in COX-2 mRNA Processing***

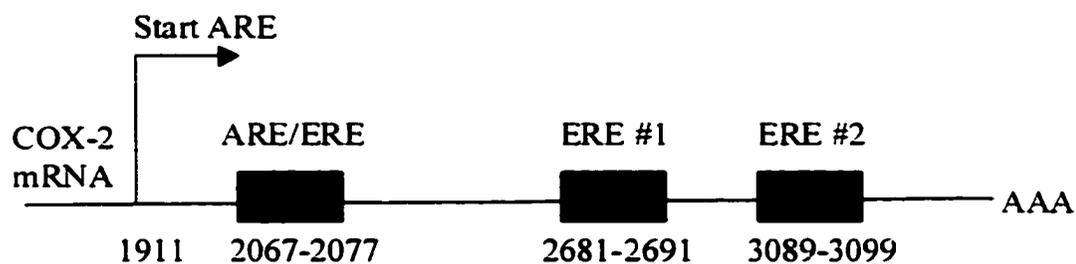
Since polyamine depletion induces COX-2 protein levels and it does not appear to be working on the level of protein stability, the next course of action was to investigate the role of polyamines on RNA stability. DFMO induced RNA steady state levels to a much smaller degree than the induction seen at the protein level. Thus, it seemed plausible that polyamines were stabilizing COX-2 mRNA to allow for increased protein synthesis. An important known mediator of RNA processing is the polyamine-dependent gene, eIF-5A.

This study hypothesized that eIF-5A was affecting COX-2 RNA stability. We identified three potential eIF-5A response elements (ERE) in the 3'-untranslated region (UTR) of the COX-2 mRNA (Figure 23A). The 3'-UTR of COX-2 is unique in that it has a AU-rich element (ARE) that consists of strings of AUUUA consensus regions (Dixon et al., 2000; Cok and Morrison, 2001). It is at these regions that K-ras mediates COX-2 RNA stability (Sheng et al., 2000), probably through increasing binding of stabilizing proteins like HuR. One ERE sequence identified overlaps with part of an ARE sequence and was thus designated as ARE/ERE. These overlapping sequences form a stem loop structure in the RNA, according to the GCG program (Genetics Consortium Group). The other two EREs were named for their order on the 3'-UTR from the 5' end of the RNA (Figure 23B).

A.

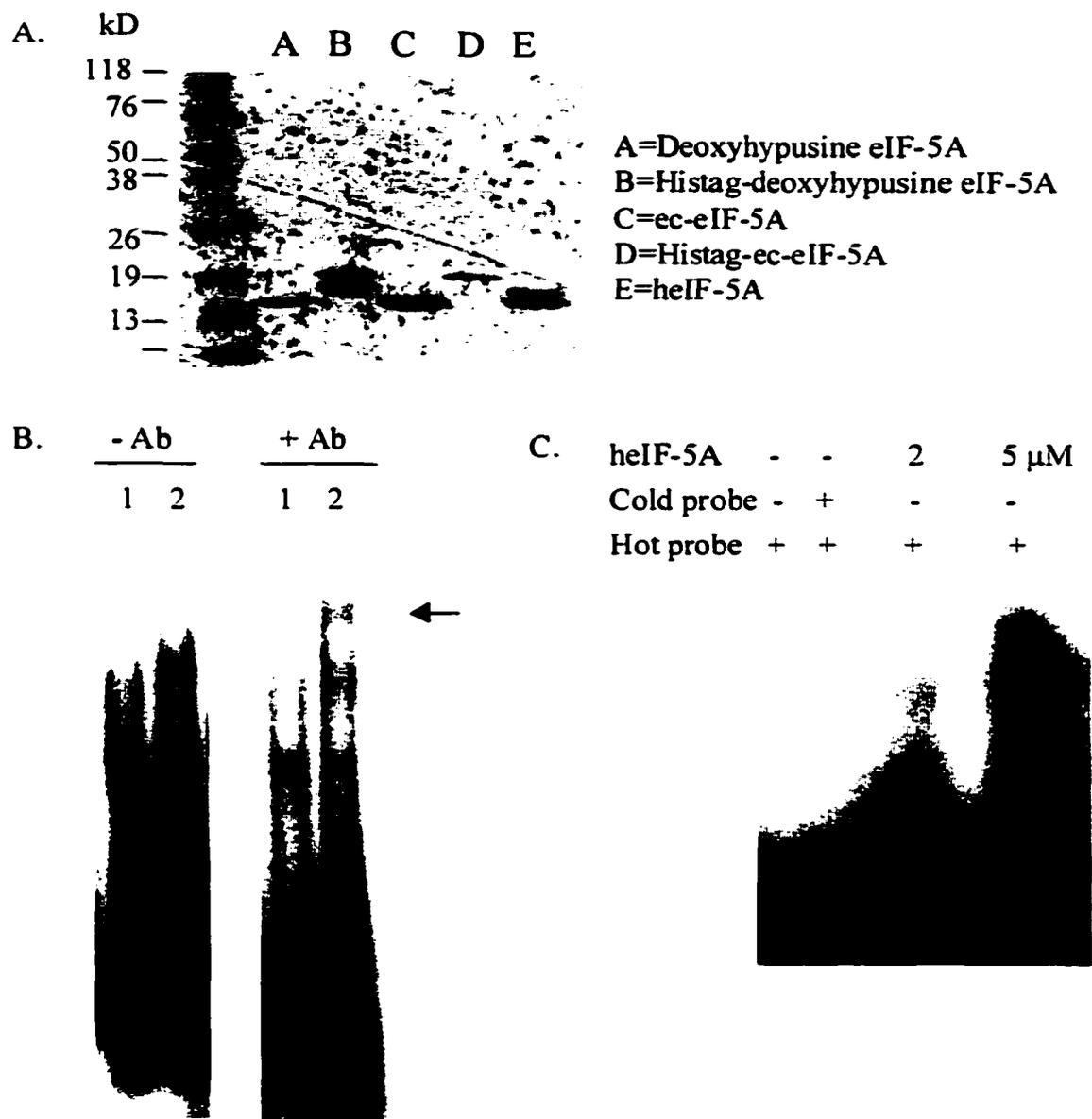
Name	Sequence	Consensus
<b>actual ERE</b>	<b>AAATGTCACAC</b>	<b>11/11</b>
ARE/ERE	* <i>TTTTA</i> TGTCAC	7/11
ERE #1	AAATGCCAAAT	9/11
ERE #2	TCATTTCACAC	8/11
*overlapping with ARE sequence= <i>AUUUA</i>		

B.



**Figure 23. eIF-5A response elements found in the COX-2 3'-untranslated region.** A. Sequence comparison of the putative eIF-5A response element (ERE) and the three EREs found in the COX-2 3'-UTR. The ARE/ERE and EREs #1 and #2 were made into  $^{32}\text{P}$ -labeled RNA probes for gel shifts using PCR and in vitro transcription. B. Location of the EREs in the AU-rich region of the COX-2 mRNA.

These ARE/ERE and ERE sequences were amplified by PCR and transcribed *in vitro* to make RNA probes for electromobility shift assays (EMSA). The probes were incubated with purified eIF-5A protein as well as extracts from colon cancer cell lines. The purified proteins were a generous gift from Dr. Myung Park and were originally run out on an SDS-PAGE gel to confirm their purity and size (Figure 24A). When human eIF-5A protein (heIF-5A) was incubated with the EREs from the COX-2 3'-UTR, a gel shift occurred (Figures 24B,C). Figure 24B shows that a partially modified eIF-5A, his-tagged deoxyhypusine eIF-5A, forms two protein-RNA complexes with the ARE/ERE and ERE #2. This may be due to an incomplete purification or partial degradation of the protein as seen by two bands in the SDS-PAGE gel in Figure 24A. When an antibody detecting His-labeled protein is added to the mixture, a supershift occurs. The fully modified human eIF-5A (heIF-5A) also binds to the ERE probe (Figure 24C).

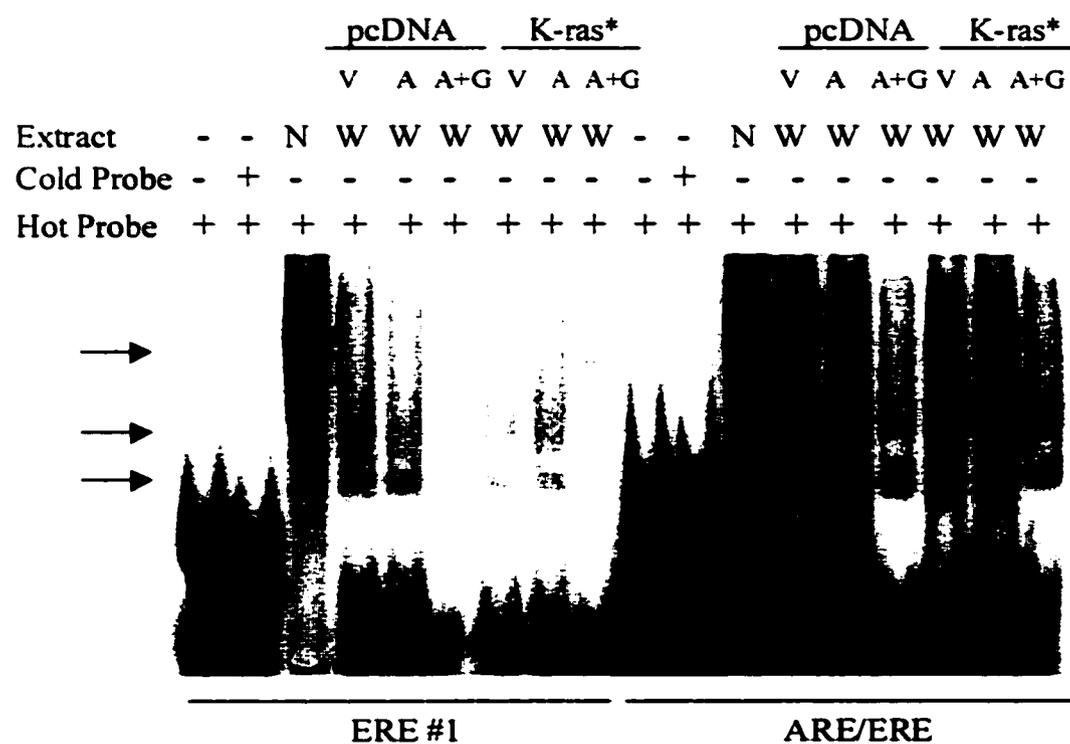


**Figure 24. Purified eIF-5A protein binds to the ERE sequences in the COX-2 3'-UTR.** A. SDS-PAGE gel of purified proteins obtained from M. Park to confirm purity and size. B. His-tagged deoxyhypusine eIF-5A was incubated with the ARE/ERE (1) or ERE#2 (2) probes and run on a nondenaturing SDS-PAGE gel. Addition of an antibody detecting His-tagged proteins caused a supershift band (arrow). C. Purified heIF-5A protein (2-5  $\mu$ M) was incubated with the  $^{32}$ P-labeled RNA probe, ERE #2. Probe was visualized by autoradiography. Cold probe competitions were used as a control for nonspecific binding. Abbreviations: Histag, protein is flagged to 6 histidine residues; ec-eIF-5A, fully unmodified eIF-5A-lysine; heIF-5A, human fully modified eIF-5A.

When the ARE/ERE and ERE probes were reacted with whole cell extract, two shifts occurred, implicating eIF-5A in two different sized protein complexes (Figure 25). With no commercial eIF-5A antibody available to conduct supershift experiments, we relied on a specific inhibitor of eIF-5A, N<sup>1</sup>-guanyl-1,7-diaminoheptane (GC-7) to verify the role of eIF-5A in these complexes. The proper concentration to use in these studies was determined by a <sup>3</sup>H-spermidine uptake experiment. The premise was that spermidine is necessary for the eIF-5A hypusine modification (see Figure 3, Chapter 1) and the <sup>3</sup>H-spermidine would only be incorporated into this particular protein, a process that would be inhibited by GC-7. Cells were pretreated with 0-100 μM GC-7 and then incubated with <sup>3</sup>H-spermidine and harvested at various timepoints. Three days of 50 μM GC-7 caused the greatest inhibition of eIF-5A (data not shown) compared to controls. Concentrations greater than 50 μM induced cell death, with the greatest amount occurring at the highest dose tested.

When cells were treated with GC-7, the binding of two protein complexes to the ARE/ERE and EREs was significantly inhibited (Figure 25). Aminoguanidine (1 mM) is used in conjunction with GC-7 to prevent degradation of the compound by cellular amine oxidases. It is interesting to notice that eIF-5A also forms protein complexes in the nuclear extracts, validating another study suggesting that eIF-5A serves a role in nucleocytoplasmic transport (Rosorius et al., 1999). The band seen in the nuclear extract is observed to a smaller degree in the whole cell extracts as well (the top arrow). This

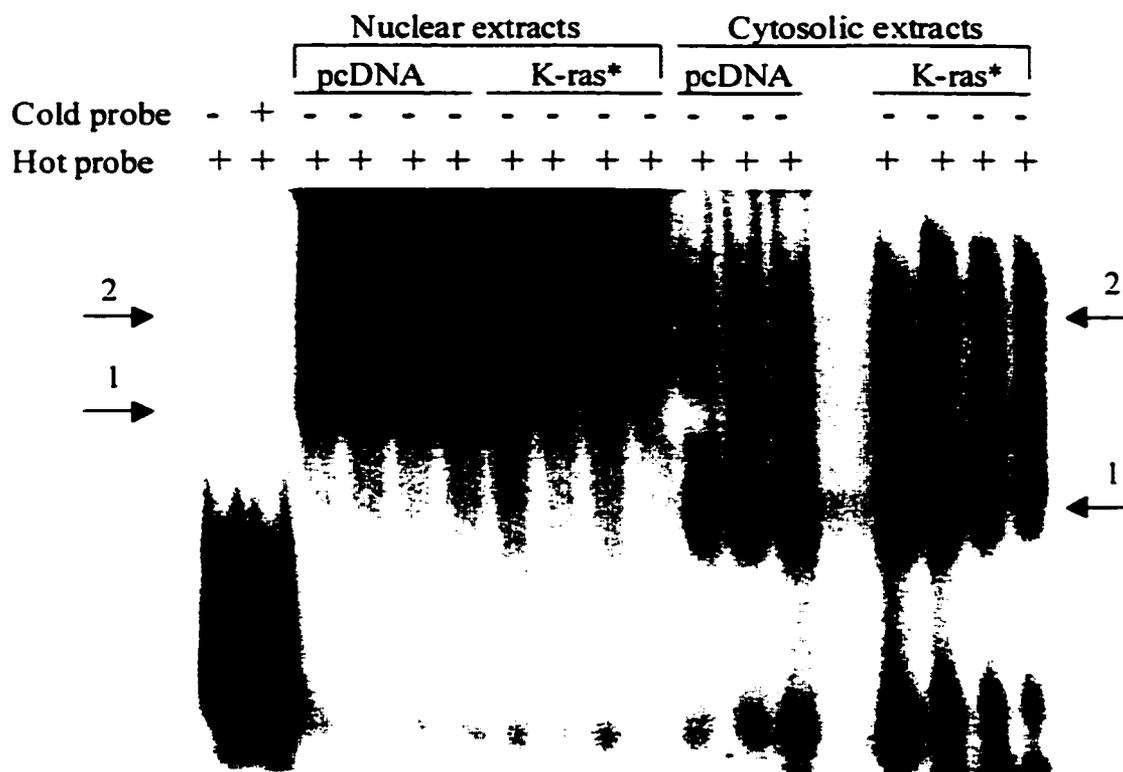
lighter band representing a larger protein complex also shows some decrease in samples treated with GC-7.



**Figure 25. Inhibition of eIF-5A by GC-7 suppresses ERE gel shifts.** Caco-pcDNA and Caco-Kras cells were treated for 3 days with vehicle (V), 1 mM aminoguanidine (A) or aminoguanidine + 50  $\mu$ M GC-7 (A+G). Cells were harvested for nuclear extract (N) or whole cell extract (W). Extracts were incubated with  $^{32}$ P-labeled

**RNA probes of ERE #1 and ARE/ERE. Cold probe competitions served as controls for nonspecific binding. Gel shift bands were visualized by autoradiography.**

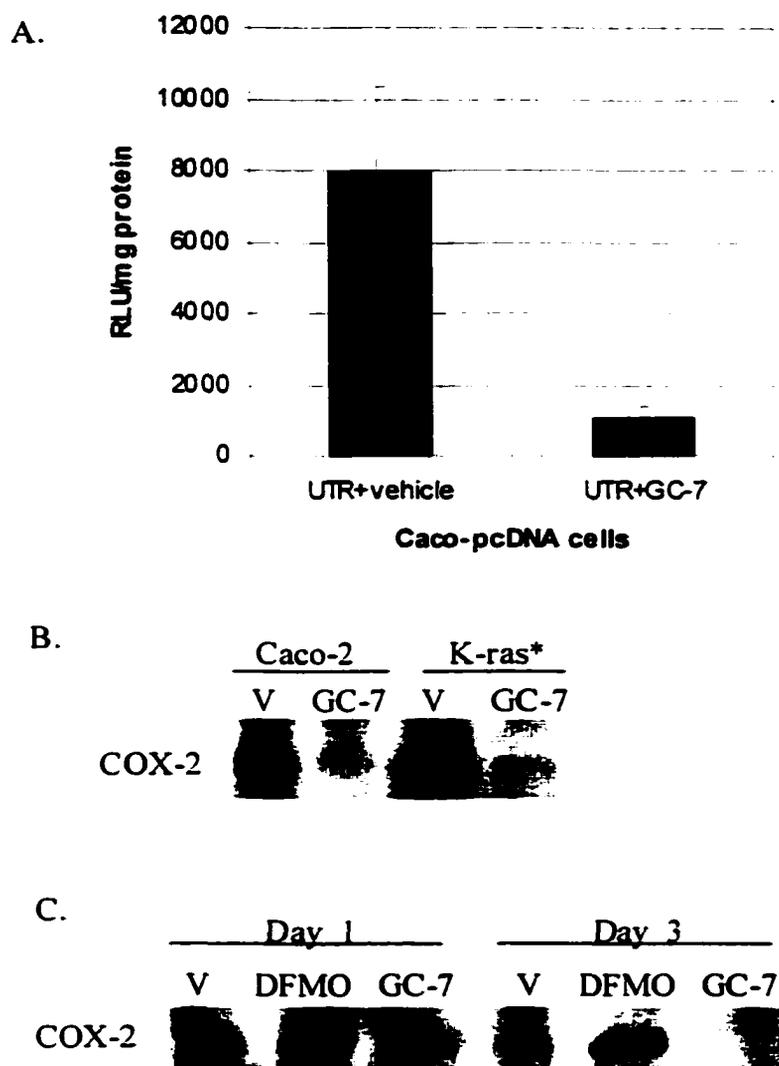
Where nuclear extracts show a gel shift band that migrates slower than the cytosolic extracts, there is also a difference in cells with a normal K-ras (pcDNA) and an activated K-ras (K-ras). A representation of this phenomenon is seen in Figure 26. With both nuclear and cytosolic extracts, K-ras activation causes a shift in the size of the protein complex binding with the EREs. Cell extracts from Caco-Kras cells form a larger protein complex with the sequences in the 3'-UTR of COX-2 than extracts prepared from Caco-pcDNA cells. Caco-pcDNA cells show a band midway down the gel, while K-ras activated cells show the same shift plus a more pronounced shift above. Overall, there appear to be three protein complexes formed. K-ras seems to be involved with the largest of these complexes.



**Figure 26. K-ras activation causes a shift in cytosolic protein complexes.** Protein extracts were prepared from nuclear and cytoplasmic fractions of Caco-pcDNA and Caco-Kras cells. Extracts were incubated with the  $^{32}\text{P}$ -labeled ARE/ERE RNA probe. Samples were run out on an SDS-PAGE gel. A cold probe competition was used to control for nonspecific binding. The (1) represents the shift caused by Caco-pcDNA cells and the (2) is the change in the shift induced by the K-ras-activated Caco-2 cells.

To further determine the effects of eIF-5A binding to ERE consensus sites on the COX-2 3'UTR, a luciferase-reporter construct was created containing the COX-2 3'-UTR downstream of the luciferase gene, driven by the CMV promoter in the pcDNA3 plasmid (Dixon et al., 2000; Sheng et al., 2000). Cells were transiently transfected with this plasmid and treated with GC-7. GC-7 reduced luciferase activity by almost 80% compared to the vehicle treated cells (Figure 27A). This suggests eIF-5A normally works to process COX-2 mRNA into protein and that GC-7 blocks that effect, resulting in less binding to the ERE and destabilization of the 3'-UTR.

The destabilization of the COX-2 3'-UTR by GC-7 leads to a decrease in COX-2 protein as well (Figure 27B). The inhibition of COX-2 protein seen with GC-7 was an opposite result to the increase seen with DFMO treatment (Figure 27C). This suggests that DFMO acts to modulate COX-2 by two distinct mechanisms - an eIF-5A-dependent mechanism because DFMO can suppress eIF-5A by inhibiting spermidine production and perhaps more importantly, an eIF-5A-independent mechanism that may actually stabilize COX-2 RNA for increased translation. This possibility is presently being further investigated.

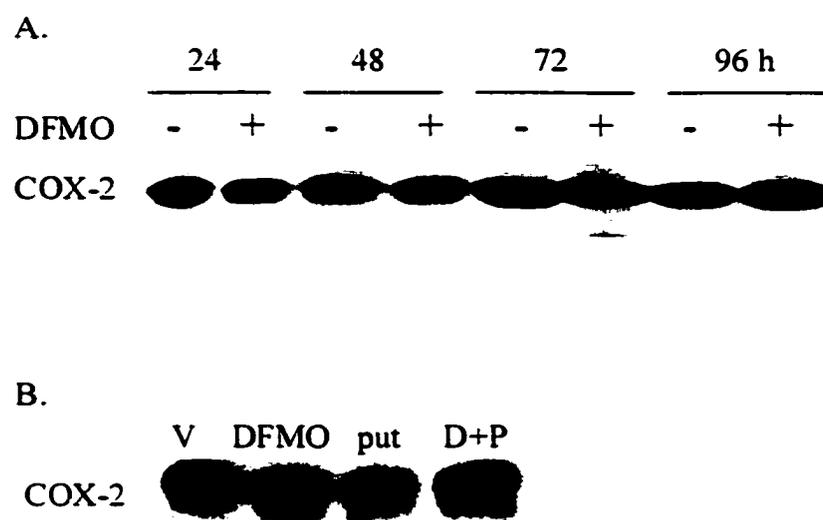


**Figure 27. GC-7 treatment destabilizes the COX-2 3'-UTR and results in decreased translation of COX-2 protein.** A. Caco-pcDNA cells were transiently transfected with the COX-2 3'-UTR-luciferase reporter construct and then treated with 50  $\mu$ M GC-7 for 2 days in the presence of 1 mM aminoguanidine. Luciferase activity was normalized to protein concentration. B. Caco-2 and K-ras activated Caco-2 cells were treated with H<sub>2</sub>O vehicle or 50  $\mu$ M GC-7 in the presence of 1 mM aminoguanidine for 3 days and harvested for COX-2 western blotting. C. Western blot analysis of Caco-pcDNA cells treated with vehicle, 5 mM DFMO or 1mM aminoguanidine + 50  $\mu$ M GC-7 for 1 or 3 days.

***HCA-7 cells exhibit a defect in polyamine metabolism***

In the process of determining the effects of DFMO treatment on COX-2 expression, several colon cancer cell lines were tested. HCA-7 cells, which are RER+ and constitutively express COX-2, were not susceptible to induction of COX-2 by DFMO (Figure 28A). COX-2 expression was also not modulated by addition of exogenous putrescine (Figure 28B).

In order to determine if HCA-7 had extremely high polyamine content compared to Caco-2 cells, which might prevent modulation by DFMO and putrescine at the concentrations used, polyamine analysis was conducted by HPLC (Figure 29A). In contrast to what we expected, the HCA-7 cells had reduced polyamine pools compared to both the Caco-pcDNA and Caco-Kras cells. The K-ras activated Caco-2 cells showed increased polyamine pools compared to the parental line, which was discovered in our lab earlier (NA Ignatenko, unpublished data). This experiment also proved that 5 mM DFMO was a high enough concentration to deplete the polyamines in the HCA-7 cells, because the spermidine pools were reduced to nondetectable levels. It is known that DFMO does not dramatically affect spermine pools (Gerner and Mamont, 1986) as spermine concentration is tightly regulated by the cells.



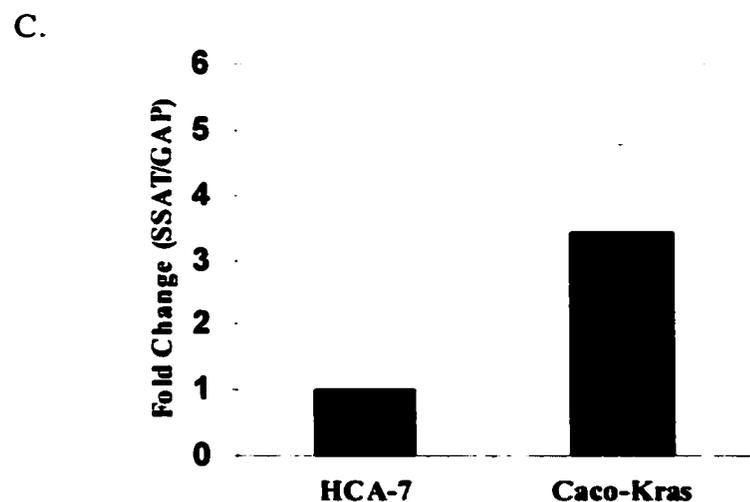
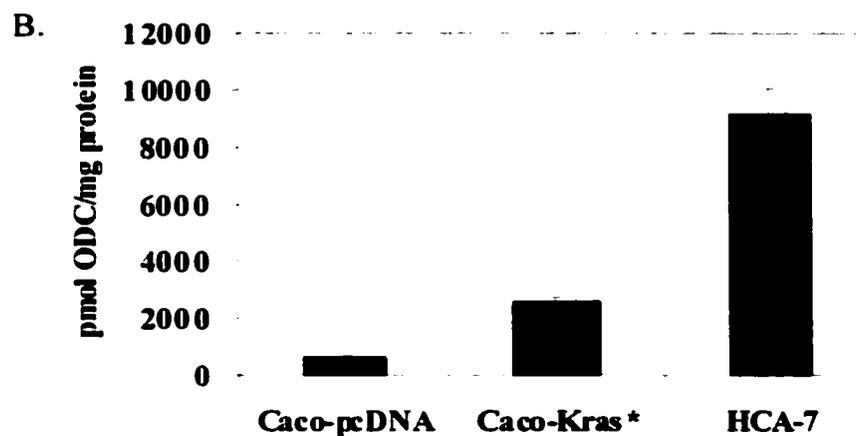
**Figure 28. HCA-7 cells are not sensitive to polyamine depletion.** A. HCA-7 cells were treated with 5 mM DFMO for 1-4 days and lysed for COX-2 western blot analysis. B. HCA-7 cells were treated with vehicle, 5 mM DFMO, 1 mM putrescine (put) or DFMO + putrescine. DFMO treatment lasted for 3 days and putrescine was added on day 2 for 24 h. Cells were lysed for COX-2 western blotting.

The discovery of reduced polyamine pools in a cell line that constitutively expresses COX-2 led us to speculate that the suppressed polyamine pools might be the causative factor in the increased COX-2 expression in this cell line. To test this hypothesis, it was important to first determine the reason for the reduced polyamine pools. The most obvious first experiment was to test ODC activity; reduced polyamine pools would be the first result of stunted ODC enzyme activity. However, HCA-7 cells have four times the ODC activity compared to Caco-2 cells (Figure 29B). K-ras activated Caco-2 cells have slightly higher ODC activity than the parental cells, leading to the increased polyamine pools seen in the previous experiment. However, an inhibition of ODC in the HCA-7 cells does not account for their low polyamine levels.

The next hypothesis tested was that the HCA-7 cells were rapidly catabolizing the polyamines to result in low intracellular levels. However, when SSAT steady state mRNA levels were measured, Caco-2 cells actually had twice the level of SSAT RNA than HCA-7 cells (Figure 29C). Thus, an overactive SSAT enzyme was not causing the low polyamine pools in the HCA-7 cells.

A.

Polyamines (nmol/mg protein)	Caco- pcDNA	Caco- Kras *	HCA-7 vehicle	HCA-7 DFMO
Putrescine	1.51	1.3	ND	ND
Spermidine	3.49	5.55	1.04	ND
Spermine	6.94	9.74	6.45	5.27
Spd/Spm Ratio	0.5	0.57	0.16	N/A



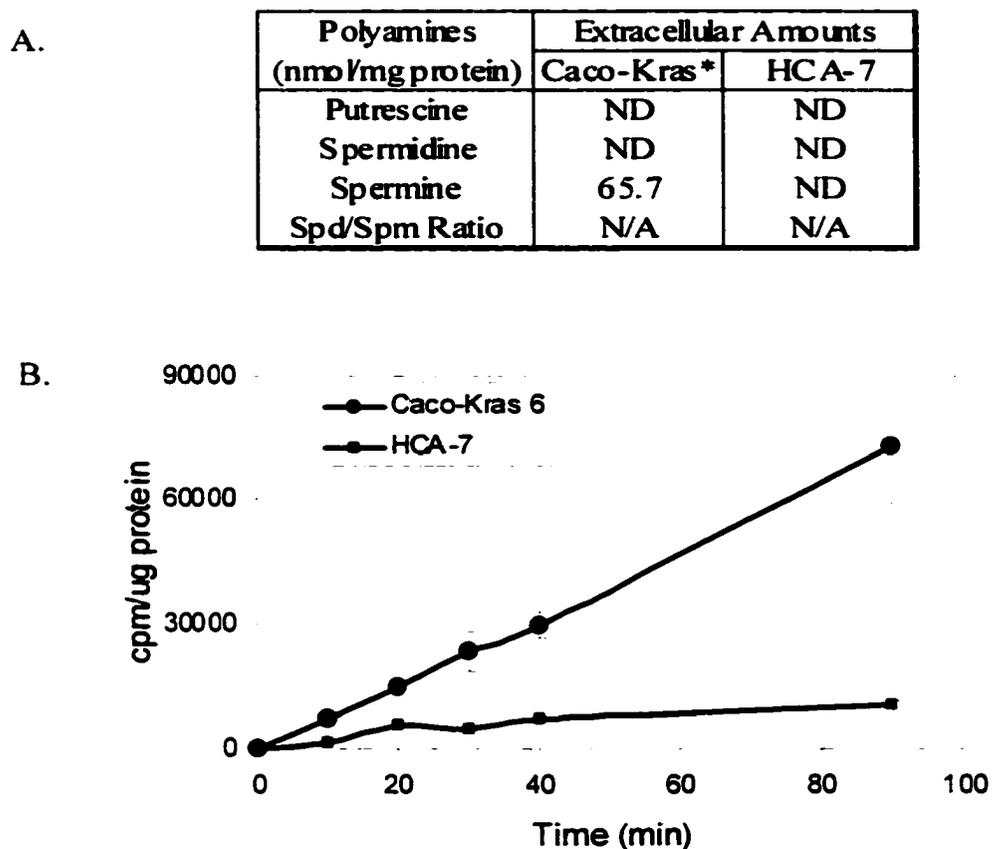
**Figure 29. HCA-7 cells have reduced polyamine pools.** A. Intracellular polyamines were measured by HPLC in Caco-pcDNA, Caco-Kras, and HCA-7 cells treated with vehicle or 5 mM DFMO for 3 days. Polyamine content was normalized to protein concentration. B. ODC enzyme activity was determined as the amount of  $^{14}\text{C}$ - $\text{CO}_2$  released during 30 min.  $^{14}\text{C}$ -ornithine was used as the substrate. Enzyme activity was normalized to protein concentration. This experiment was performed in triplicate. C. SSAT mRNA steady state levels for HCA-7 and Caco-Kras cells normalized to GAPDH (GAP) mRNA as a loading control.

***HCA-7 cells have a defect in polyamine transport***

Polyamine pools are tightly regulated in colonic epithelial cells and are not solely dependent upon biosynthetic and catabolic enzyme levels. Thus, to further investigate the defect in polyamine metabolism in the HCA-7 cells, experiments were conducted to determine the amount of polyamine transport across the cell membrane. Extracellular polyamines were measured by incubating the cells with a small volume of serum-free media for 1-4 hours to concentrate the polyamines being exported from the cells over that time period. The media was then harvested and analyzed for polyamine content. This experiment was the first to suggest that HCA-7 cells have a defect in polyamine transport as they did not export any of the three main polyamines out the cell (Figure 30A). Caco-2 cells, on the other hand, exported copious amounts of spermine into the media during this same time period. It is not known why the Caco-2 cells export such high amounts of spermine or why there is no detectable putrescine or spermidine. The extracellular amounts of putrescine and spermidine might be below the level of detection of our assay. Regardless of the reason, the extracellular polyamine profile looks very different in the HCA-7 cells compared to the Caco-2 cell model.

Since the HCA-7 cells exhibited a defect in polyamine export, we questioned if they also showed difficulty with polyamine import. This proposition was tested by incubating cells with  $^3\text{H}$ -putrescine and harvesting cells at various timepoints. The radioactive media was thoroughly washed off from intact cells and the amount of  $^3\text{H}$ -putrescine inside the cells was determined by scintillation counting. The rate of polyamine uptake was much greater in the Caco-2 cells than in the HCA-7 cells (Figure

30B). By 30 min, the Caco-2 cells had already imported 2.5 times the amount as the HCA-7 cells. By the end of the experiment, the Caco-2 cells had 5 times the intracellular  $^3\text{H}$ -putrescine content than the HCA-7 cells. Thus, it can be concluded that the defect in polyamine metabolism seen in the HCA-7 cells is a problem with polyamine transport both in and out of the cell.



**Figure 30. HCA-7 cells have a defect in polyamine transport.** A. Extracellular polyamine content was measured on the HPLC from serum-free medium on the Caco-Kras and HCA-7 cells. B. Caco-Kras (clone 6) and HCA-7 cells were incubated in medium containing <sup>3</sup>H-putrescine for the times indicated and then washed thoroughly. <sup>3</sup>H-putrescine uptake was determined using a scintillation counter. Counts per minute (cpm) were normalized to protein concentration. The error bars signify triplicate samples.

## **Summary**

The pharmacological depletion of polyamines by DFMO greatly induced COX-2 protein expression in a time-dependent manner (Figure 19). This was shown to begin at the level of RNA, but did not affect promoter activity (Figures 20-21). However, the effect of DFMO on steady levels of mRNA was much smaller than that seen at the protein level. This data led us to investigate possible roles of polyamines in COX-2 mRNA and protein stability. There was no effect of the polyamine-regulated antizyme protein on COX-2 degradation, but modulation of the polyamine-regulated eIF-5A protein significantly affected COX-2 protein levels, implicating RNA stabilization as mechanism for DFMO-induced COX-2. However, this does not rule out the possibility that polyamines may also be regulating COX-2 translation initiation or elongation.

Inhibition of eIF-5A by GC-7 resulted in decreased binding of eIF-5A-associated protein complexes to the EREs in the COX-2 3'-UTR. This effect was observed both in RNA gel shifts and luciferase-reporter assays (Figures 25, 27A). The effects seen with GC-7 are specific to eIF-5A as illustrated by the ability of GC-7 to prevent <sup>3</sup>H-spermidine uptake. Inhibition of eIF-5A by GC-7 also led to suppression of COX-2 protein as seen by western blots (Figure 27B). This piece of data leads to the conclusion that polyamines are regulating COX-2 by two distinct mechanisms. Firstly, DFMO inhibits polyamine production, resulting in decreased spermidine pools. This will negatively affect eIF-5A modification and binding to the ERE consensus sites on COX-2. If the function of eIF-5A is to help process COX-2 mRNA into protein, then DFMO will inhibit protein synthesis

in this scenario. However, as shown in Figures 19 and 27C, DFMO significantly increases COX-2 protein expression. Thus, DFMO and polyamines must be acting on COX-2 by an eIF-5A-independent mechanism as well.

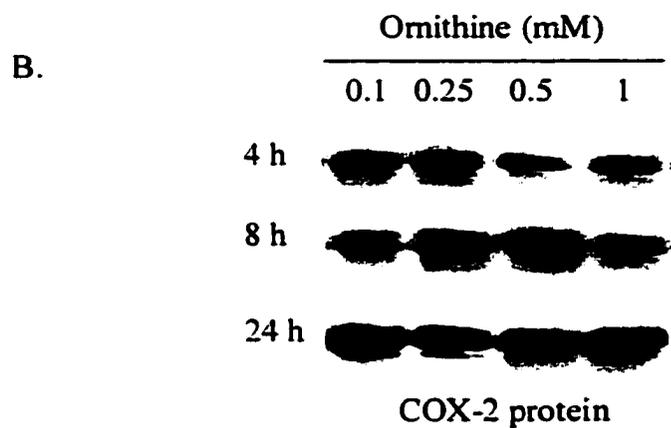
We have evidence that this other mechanism is not at the transcriptional level as DFMO does not affect COX-2 promoter activity and for that matter, only has a small effect on steady state mRNA levels. So DFMO must mediate COX-2 RNA stability, translation efficiency, or protein stability. According to experiments with the transcription inhibitor, actinomycin D, and pulse-chase experiments, DFMO does not work to stabilize COX-2 mRNA or protein (data not shown), but we have not yet ruled out effects on translation initiation or efficiency. DFMO may also be affecting RNA transport out of the nucleus, thereby affecting eIF-5A's ability to process it into protein. This would suggest a dual role of polyamines in eIF-5A-mediated COX-2 expression. Polyamines, spermidine specifically, could induce eIF-5A modification, and then polyamines could inhibit the nucleocytoplasmic trafficking of the eIF-5A-processed COX-2 RNA. Thus, a decrease in polyamines by DFMO would suppress this inhibition and cause greater trafficking out of the nucleus and increased translation of COX-2 protein, which correlates to the increased protein expression seen by western blot. This hypothesis is currently being investigated with slot blot experiments to see if DFMO affects the exchange of nuclear to cytoplasmic pools of COX-2 mRNA. There is evidence in the literature for polyamines mediating  $\text{Ca}^{2+}$  movement in and out of the mitochondria (Lenzen et al., 1986), so it conceivable that polyamines may be trafficking other molecules out of other organelles, like RNA out of the nucleus.

In the case of the HCA-7 cells which constitutively express COX-2, polyamine transport in and out of the plasma membrane appears to be stunted. The intracellular polyamine pools are much lower than in the Caco-2 cells, yet they do not have reduced ODC activity or increased SSAT mRNA levels. The main difference in the two cell lines is polyamine import and export from the extracellular environment. HCA-7 cells do not export any of the three main polyamines, while the Caco-2 cells export large amounts of spermine. Also, HCA-7 cells have a stunted rate of  $^3\text{H}$ -putrescine import compared to the Caco-2 cells.

It has not yet been proven that this defect in polyamine transport is the reason for the constitutive levels of COX-2 in this cell line. However, one piece of evidence that polyamines are regulating COX-2 in this cell line is an ornithine-dependent inhibition of COX-2. When HCA-7 cells are exposed to exogenous ornithine, it appears that they are able to import it and use it to synthesize polyamines as the intracellular polyamine levels increase significantly (Figure 31A). At the same time, COX-2 protein expression is inhibited (Figure 31B). This does not necessarily prove that the polyamine induction causes the COX-2 reduction, but it is suggestive of that possibility. Further study of the regulation of COX-2 by polyamines is necessary to properly discern the mechanism.

A.

Polyamines (nmol/mg protein)	HCA-7 cells	
	vehicle	Ornithine
putrescine	ND	8.2
spermidine	2.4	7.3
spermine	6	19.7



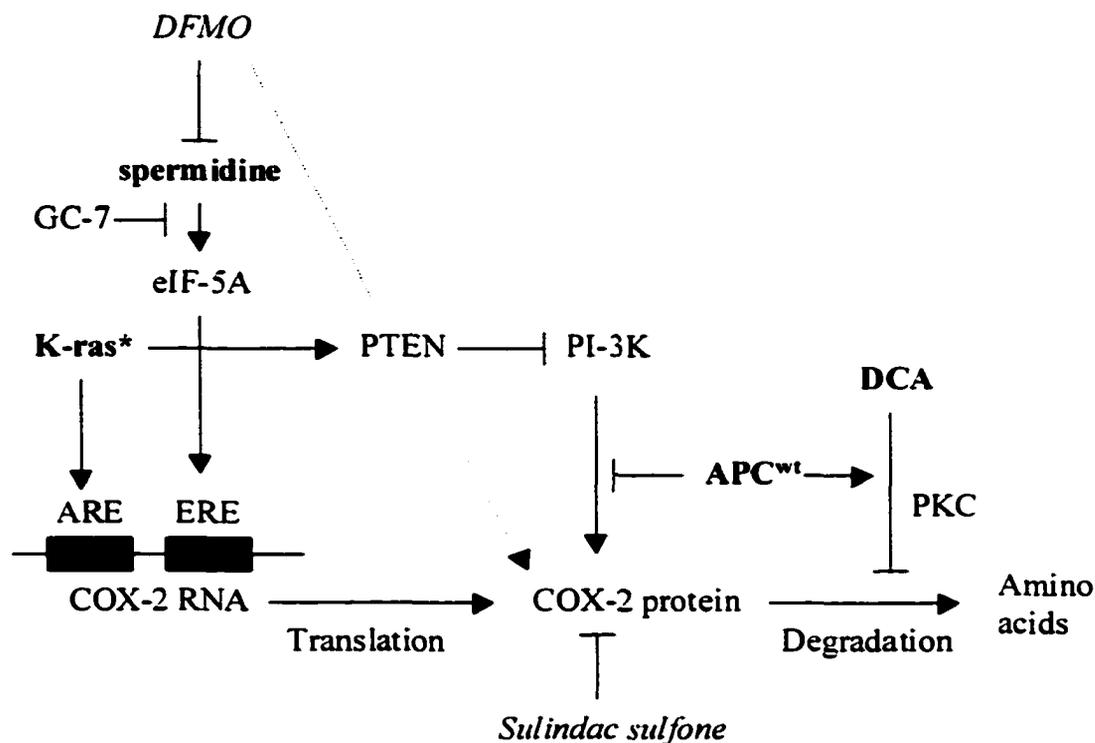
**Figure 31. Exogenous ornithine increases polyamine pools and decreases COX-2 protein expression.** A. HCA-7 cells were treated with 1 mM ornithine for 4 h before harvesting for polyamine analysis. B. HCA-7 cells were treated with 0.1-1 mM ornithine for 4-24 h and lysed for COX-2 western analysis.

## **CHAPTER 7: DISCUSSION AND PERSPECTIVES**

The delicate balance of oncogenes promoting growth and tumor suppressor genes inhibiting growth serve to regulate development and apoptosis of tissues in the human body. When this balance becomes disrupted, it is often the beginning of the tumorigenesis process. The studies in this dissertation (see Figure 32 for summary diagram) evaluated the mechanisms by which oncogenes and tumor suppressor genes regulate the COX-2 enzyme, whose activity is greatly increased in colonic adenomas and carcinomas. Two common mutations in colon cancer, an activated K-ras and a truncated APC both acted to induce COX-2 expression in our human colon cancer cell lines. K-ras mutation led to an increase in COX-2 at the posttranscriptional level in cells with functional PKC signaling. A wild-type APC was necessary for the K-ras-mediated induction of COX-2 in cells with dysfunctional PKC signaling. K-ras induced COX-2 through PI-3K-dependent signaling, which could be blocked with wild-type APC. The participation of multiple signaling pathways predicts the existence of crosstalk between these pathways. We show that these signal transduction cascades operate in a cell-specific manner, and sometimes irrespective of mutational status, as seen in the comparison of the Caco-2 and HT-29 cell models. It is crucial to understand these pathways in depth as they may prove to be excellent targets for both chemoprevention of aberrant cells or targeted chemotherapies against the cancerous cells.

In addition to genetic mutations, the effects of intestinal luminal contents on COX-2 expression was investigated, concentrating on two dietary factors: bile acids and polyamines. The secondary bile acid, DCA induced COX-2 in a APC-dependent manner in some cell lines yet was unable to induce COX-2 in cell lines with disrupted PKC signaling. This was just one example from this dissertation on how genetics and downstream signals interact with environmental factors (dietary risk factors) to regulate COX-2 and tumorigenesis. Whereas K-ras and APC regulate COX-2 posttranscriptionally, DCA regulates COX-2 at the level of protein stability.

Polyamines are another luminal risk factor for colon cancer and never before had an interaction between them and COX-2 been established. Chapter 6 of this dissertation showed that polyamines downregulate COX-2 protein expression without affecting transcription. The polyamine-dependent gene, eIF-5A was shown to mediate processing of COX-2 message into protein, a process that could be specifically abrogated by the eIF-5A inhibitor, GC-7. In contrast, DFMO, which would decrease spermidine pools to inhibit eIF-5A caused a significant induction in COX-2 protein, suggesting the existence of an eIF-5A-independent mechanism of action as well.



**Figure 32. Cumulative summary of COX-2 regulation by genetic and luminal risk factors.** K-ras, APC, bile acids and polyamines can all regulate COX-2 at the posttranscriptional level in human colon cancer cell lines. K-ras mediates COX-2 RNA stability through AREs and polyamines promote COX-2 RNA processing through eIF-5A. Inhibition of this process by DFMO can also induce COX-2 protein expression. Wild-type APC inhibits PI-3K-dependent signaling of COX-2 protein and also potentiates bile acid-mediated COX-2 protein stability. The chemopreventive agents, *Sulindac sulfone* and *DFMO* have opposite effects on COX-2 protein. Genetic and luminal risk factors are in **bold** and chemopreventive agents are in *italics*.

Further studies are underway to elucidate how this chemopreventive agent is actually inducing COX-2 levels irrespective of its effects on eIF-5A. Our studies have shown that polyamine depletion causes an induction of COX-2 RNA and protein expression. A decrease in eIF-5A modification causes a decrease in COX-2 protein. In addition, we are presently generating data showing that polyamines may be influential in RNA aggregation, creating a nontranslatable message. This phenomenon could potentially be reversed with DFMO treatment, resulting in the observed increase in COX-2 RNA and protein. eIF-5A may work to counter RNA aggregation, producing a translatable message. This potential model would explain how the inhibition of eIF-5A with GC-7 yields opposite results as polyamine depletion by DFMO.

The observation that DFMO increases COX-2 protein in a time-dependent fashion underscores the importance of using combination chemoprevention strategies. If DFMO alone was used, polyamine biosynthesis would be suppressed, but at the same time, COX-2 protein would be elevated, perhaps combating the anti-cancer effects of DFMO alone. If sulindac or other COX enzyme inhibitor was used alone, the patients would not get the added benefit of polyamine depletion. The two metabolites of sulindac have drastically different effects on COX-2 protein, even though both the sulfide and sulfone metabolites abate COX enzyme activity. For the first time, these studies show that sulindac sulfone can inhibit K-ras-mediated COX-2 protein expression, which could have the downstream effect of interfering with signal transduction pathways. These data help to elucidate the molecular biology behind the mechanism of action of these

chemopreventive agents, which will hopefully lead to the development of new agents and new combinations to suppress tumorigenesis.

The studies represented in this dissertation highlight the importance of combination chemoprevention - not simply two agents, but perhaps multiagent interventions. The mechanisms by which genetic mutations and carcinogens from dietary intake affect carcinogenesis are numerous and complex. To fully combat the carcinogenic process, many interventions at various stages may be necessary. In addition, each patient's risk for colon cancer is individual. Patients at high risk for disease due to family history may want to begin chemoprevention early in life. Patients who eat healthy, low-fat diets may need less intervention, perhaps only a single agent like a COX-inhibitor or antioxidant to maintain their low risk for disease. If genotyping of the general public becomes easy and popular, each patient's chemoprevention regimen could be tailor-made for his/her genetic make-up and lifestyle. Or a primary polyp could be genotyped to narrow in on the most beneficial intervention to prevent recurrence. The key to better interventions is the elucidation of the biological interactions between a variety of risk factors for cancer.

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