This is to certify that the
dissertation entitled

BLACK BEANS AND SOY FLOUR INHIBIT EXPERIMENTAL COLON CARCINOGENESIS BY REDUCING INFLAMMATION AND ENHANCING COLONOCYTE DIFFERENTIATION

presented by

ELIZABETH ANN RONDINI

has been accepted towards fulfillment
of the requirements for the

Doctoral degree in Human Nutrition

Maurice R. Bennink
Major Professor's Signature

5-8-06
Date

BLACK BEANS AND SOY FLOUR INHIBIT EXPERIMENTAL COLON CARCINOGENESIS BY REDUCING INFLAMMATION AND ENHANCING COLONOCYTE DIFFERENTIATION

By

Elizabeth Ann Rondini

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science and Human Nutrition

2006

ABSTRACT

BLACK BEANS AND SOY FLOUR INHIBIT EXPERIMENTAL COLON CARCINOGENESIS BY REDUCING INFLAMMATION AND ENHANCING COLONOCYTE DIFFERENTIATION

By

Elizabeth Ann Rondini

Colorectal cancer (CRC) is one of the most common neoplasms afflicting Western societies (1). The geographical differences in CRC and studies in migrant populations provide strong evidence that a majority of sporadic CRC cases are attributable to environmental exposures, more specifically dietary habits (2-4). Legumes (peas, beans, peanuts, lentils) are among dietary components frequently consumed by populations who have a lower risk for cancers of the colon, breast, and prostate (7). Experimental studies conducted in animals have also demonstrated that bean-based diets inhibit the development of colonic neoplasms (14-18, 265). Because diet is a major determinant of risk for CRC, this research was designed to achieve a more thorough understanding of cellular and molecular events involved in chemoprevention by beans using the azoxymethane (AOM)-induced rodent model of colon cancer.

The first experiment was conducted to assess biological processes involved in cancer promotion. Microarrays were performed to profile genes differentially expressed in colon tumors compared to non-neoplastic colonic mucosa in rats treated with the carcinogen AOM. A majority of genes affected during cancer development were related to immune responses and inflammation, extracellular matrix remodeling, and suggestive of global disturbances in cellular metabolism and epithelial ion transport. Having determined genetic alterations associated with cancer promotion, the second experiment

was designed to identify molecular targets permissive for tumorigenesis and those associated with cancer inhibition by bean-feeding. Microarrays were performed on mRNA isolated from normal-appearing colonic mucosa in rats treated with either AOM or saline and fed a control (casein), black bean (BB), or soy-flour (SF) based diet for 30 weeks. Carcinogen (AOM) treatment was found to induce expression of genes involved in inflammatory and immune responses, protein synthesis, and extracellular matrix remodeling. BB- and SF-fed rats exhibited a higher expression of genes involved in energy metabolism and a lower expression of inflammatory and cell cycle-associated genes. Genes involved in extracellular matrix (collagen 1α1, fibronectin 1) and in innate immunity (NP defensin 3α, secretory phospholipase A2 (sPLA2)) were induced by AOM in all diets, but less so in bean-fed animals. This gene profile suggests that bean diets inhibit colon cancer by maintaining crypt cell homeostasis and reducing inflammation.

The third study was designed to address whether dietary differences in sPLA2 were associated with alterations in colonic epithelial proliferation and inflammation in colon tissue using immunohistochemical techniques. Rats treated with AOM and fed a control diet were found to have a higher zone of proliferative cells, a higher content of sPLA2, and evidence of infiltrating macrophages in colonic tissue compared to rats fed BB, SF, or control-fed animals not administered carcinogen. A positive and significant correlation was found between sPLA2 immunoreactivity and the zone of proliferative cells. These data, consistent with microarray profiles suggest that the ability of beans to suppress colon cancer occurs early in the neoplastic process and is associated with modulating inflammation and enhancing cellular differentiation.

DEDICATION

To my family, especially my parents, for their love, encouragement, and support at all times. Thank you for everything... you mean the world to me.

ACKNOWLEDGEMENTS

I would like to thank my major advisor, Dr. Bennink, for his support and guidance throughout my graduate studies. I would also like to extend my gratitude to Dr. Romsos and my committee members, Dr. Bourquin, Dr. Claycombe, and Dr. Orth, for their useful suggestions and review of my dissertation, and to Annette Thelen for technical assistance with microarrays. I would also like to thank my parents for their support, both emotionally and financially, which has made my education both possible and meaningful. Lastly a "shout out" to my best friends Joy Dubost and Mary Przepiora, sister Nicole "Bangs", and friends and colleagues Kathleen Barrett, Janette Harkins, Inese Cakstina, Neil Birmingham, and Bing Wang for their assistance with my research, encouragement, and for all the good times.

TABLE OF CONTENTS

LIST OF TABLES

APPENDICES.

LIST OF FIGURES

CHAPER II.

CHAPTER III.

CHAPTER IV.

CHAPTER V.

CHAPTER I.

INTRODUCTION

Colorectal cancer (CRC) is the one of the most common neoplasms afflicting industrialized societies (1). In 2002, there were 528,978 deaths due to colorectal cancer worldwide, with 59,345 cases in the United States alone (1). Both genetic and environmental exposures have been implicated in the etiology of CRC, and up to 75% of cases may be preventable by adequate diets and regular exercise (2-4). Generally, consumption of diets low in red meat and alcohol and high in vegetables and cereal grains is associated with a decreased risk of developing CRC (2-4). Results from several epidemiological studies also indicate that populations consuming higher intakes of legumes (peas, beans, lentils, peanuts) have a lower occurrence of (5-6) and mortality from CRC (7). Legumes are naturally high in dietary fiber, micronutrients, and several bioactive compounds (*ie.* phytoestrogens, protease inhibitors, saponins, phytic acid) that may contribute to anti-cancer properties (270-276, 280-281). However the mechanisms underlying reduced susceptibility to colon cancer development have not been fully elucidated.

The development of colon cancer from normal epithelium is a genetically driven process (8-11). Several genes altered during the adenoma-carcinoma sequence in humans have been identified (8-12), and have led to a more thorough understanding of molecular events important in the pathogenesis of CRC. With the advent of technology available to explore multiple genes simultaneously, it is now possible to determine how certain foods and/or food components influence colon carcinogenesis at a cellular level.

Several animal models have been developed to examine various aspects of colon cancer under controlled conditions (13). Among these, the azoxymethane (AOM)-induced rodent model is one of the most widely used models for examining dietary

influences on colon cancer development. In this model, feeding either dry beans (14-15) or soy flour (16-18) inhibits the development of carcinogen-induced colon tumors. Therefore, the purpose of the current research was to identify early cellular events associated with colon cancer inhibition by beans *in vivo* using oligonucleotide microarrays. Histochemical procedures were then performed to assess diet-related differences in colonic epithelial kinetics and inflammation.

CHAPTER II.

LITERATURE REVIEW

A. INCIDENCE AND ETIOLOGY OF COLORECTAL CANCER.

Colorectal cancer (CRC) is one of the most common types of neoplasms, with an estimated total 1,023,152 cases diagnosed worldwide in 2002 (1). The highest rates occur in more developed regions such as North America, Western Europe, and Australia contributing to 65% of the total global incidence of CRC (1,2). In the United States, CRC remains the fourth most common type of cancer and, after lung cancer, is the second leading cause of all cancer deaths (19). Incidence rates are slightly higher in men than women, with a predominance of cancers arising in the distal colon (30%) and rectum (29%) and the remainder (39%) in the proximal colon (20).

Both genetic and environmental factors have been implicated in the etiology of CRC. The inherited syndromes familial adenomatous polyposis (FAP) and heredity non-polyposis colorectal cancer (HNPCC) account for approximately 5-10% of cases and up to 25% may arise from genetic susceptibility factors that have not yet been fully elucidated (21). The remainder of cases (65-70%) arise sporadically, with over 90% of new CRC cases diagnosed in individuals greater than 50 years of age (11, 19, 21). The geographical differences in CRC and studies in migrant populations provide strong evidence that a majority of sporadic cancers are attributable to environmental exposures (2). More specifically, dietary and lifestyle habits are thought to contribute to most of the worldwide variation in the incidence of CRC (2). Generally, populations at increased risk for the disease consume higher intakes of red and processed meat and alcohol and lower intakes of cereals, dietary fiber, and vegetables (2-4). Other factors related to an increased risk include obesity (4, 22-25), insulin resistance (25-28), and ulcerative colitis (29) whereas physical activity (4, 30), nonsteroidal anti-inflammatory drugs (NSAIDs)

(4, 31), and, in women, hormone replacement therapy are inversely associated with CRC (4, 32).

B. PATHOGENESIS OF COLORECTAL CANCER.

B.1 Normal Histology.

The human colon is approximately 150 cm (5 ft.) in length and is functionally important for water and electrolyte absorption and evacuation of stool (33-34). The surface of the colon is composed of a single layer of epithelial cells, termed the mucosa, that extend downward into the lamina propria forming crypts (35-36). The epithelial lining is maintained by a population of multipotent stem cells anatomically located in the base of the crypts (35-36). Cell division is normally confined to lower 1/3 of the crypts and as cells migrate towards the lumen, they terminally differentiate into absorptive, goblet, or enteroendocrine cell types (35-36). Once cells reach the crypt apex, they undergo apoptosis and are shed into the lumen. In humans, the entire surface of the colon is replaced every 3-6 days (35).

B.2 Histopathology.

The development of colorectal cancer from normal appearing epithelium is associated with alterations in cell proliferation, differentiation, apoptosis, and cellular senescence. These changes are genetically driven and for a majority of CRC (>90%) manifest through distinct histological steps (37-39). An early event in the process of colon carcinogenesis involves an expansion of the proliferative zone towards the lumen (37-38). This hyperproliferative change precedes tumor development and has been identified throughout the colon of humans predisposed to colon cancer and in rodents

administered carcinogen (37, 40). Eventually, either through gene mutation or deletion, there is a focal defect resulting in an abnormal accumulation of proliferative cells (polyp) within a crypt. Two types of polyps can be distinguished histologically. Hyperplastic (nondysplastic) polyps are more common, containing cells with normal morphology aligned in a single layer along the basement membrane, and generally not considered to have malignant potential (35). Comparably, adenomatous (dysplastic) polyps contain epithelial cells with abnormal morphology, arranged in several layers along the basement membrane, and are more likely to progress to cancer (35). As adenomatous polyps grow in size the epithelial lining invaginates and expands in various directions, forming first a tubular than villus structure (11, 35, 38). Transition to malignancy is accompanied by a progressive increase in dysplasia followed by the potential of cells to invade the basement membrane and metastasize to distant organs (11, 35, 38).

Although polyps are the earliest macroscopic lesions detectable, aberrant crypt foci (ACF) have been proposed to precede adenoma development. ACF are microscopic lesions usually composed of crypts that are larger in size, have altered lumenal openings, and exhibit thickened epithelia (41-43). ACF are found in colons of rodents injected with carcinogen and in humans at increased risk of colorectal cancer. Although ACF contain similar genetic alterations to those in human cancers, a large portion of ACF are not dysplastic (42) and, in animals, ACF do not consistently correlate with adenocarcinoma development (43-44). Therefore the proposed role of ACF as precursors to adenomas remains elusive.

B.3 Molecular genetics.

Carcinogenesis is considered to be a multi-step process, with each step representing mutations, deletions, and/or epigenetic alterations in genes normally regulating cell growth and behavior (11). Two classes of genes are commonly affected in neoplasia. Tumor suppressor genes normally restrain cell growth and inactivation of both alleles results in a "loss of function" phenotype. Conversely, proto-oncogenes promote normal cell growth and mutations cause constitutive activation (45). Each mutation confers the cell with a selective growth advantage accompanied by a progressive change from normal to malignant behavior (11, 46). For each tissue, the mutations and order that they occur comprise the genetic pathway (11).

More than 10 years ago, Fearon and Vogelstein proposed the genetic model for colorectal cancer development based on mutational analyses of tumors at various stages of the adenoma-carcinoma sequence (8-10). According to this model, colorectal cancer arises from the cumulative loss of functional tumor suppressor genes and dominant activation of proto-oncogenes, with the former predominating (9-10). Somatic mutations in at least 4-5 genes are necessary for malignant transformation, whereas fewer are associated with benign tumor growth. Further, genetic alterations occur in a preferred sequence, with only a subset of genes capable of initiating the tumorigenic process (8-10). The most common mutational events include loss of function of the adenomatous polyposis coli (*APC*) gene, *p53* gene, one or more genes on chromosome 18q, and constitutive activation of *k-ras* **(FIGURE 1)**.

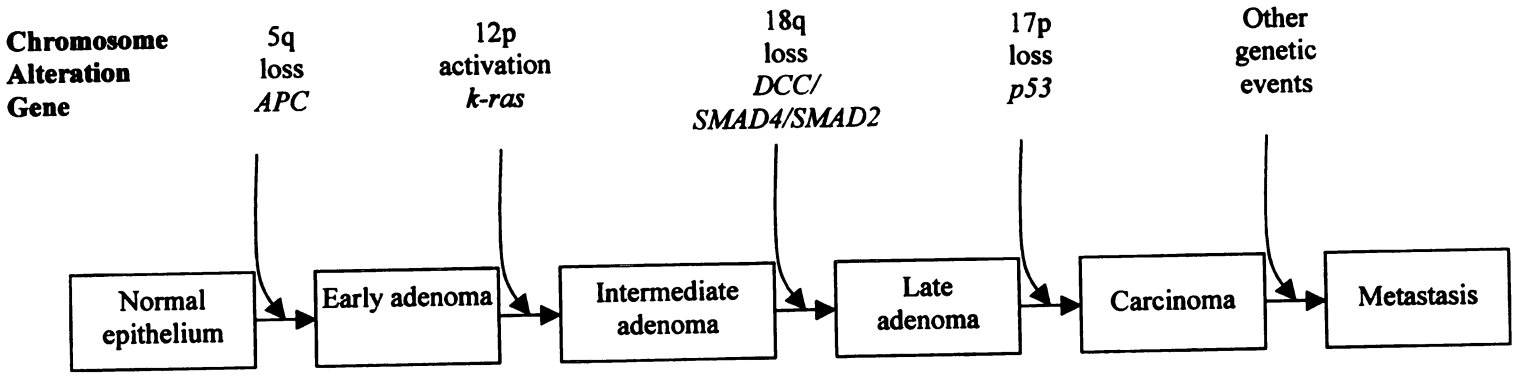


FIGURE 1. Genetic alterations in colon cancer.
Adapted from references 8-10.

B.3.1 *APC.*

APC, located on chromosome 5q21 in humans, encodes a protein with tumor suppressive properties (10). Inactivation of *APC*, either through mutation or allelic loss is permissive for adenoma formation and has been detected in 60-80% of sporadic colon tumors (47-48). Analysis of the *APC* gene from sporadic and inherited forms of colon cancer indicate that mutations occur most frequently in codons 1286-1513, named the mutation cluster region (MCR) (48-50). More than two-thirds of mutations result in frameshift, and one-third involve nonsense point mutations, both resulting in premature truncation of the polypeptide (49). Functional regions downstream of the MCR include those involved in down regulation of β-catenin and binding sites for interaction with microtubules, EB1, and DLG, suggesting these regions may all be involved in the tumor suppressive properties of APC (49, 50).

The involvement of APC in controlling intracellular levels of β-catenin has been one of the most widely researched areas in colon tumorigenesis. β-catenin associates with cadherins in the plasma membrane and also exists in a monomeric (free) form in the cytoplasm, which, upon stimulation, functions in a signal transduction pathway (51, 52). In the absence of Wnt signaling, the cytosolic level of β-catenin is tightly controlled (45). Glycogen synthase kinase (GSK) 3β, a serine/threonine kinase, phosphorylates both β-catenin and APC (45, 49, 53) and phosphorylation results in an APC/β-catenin/GSK-3β/axin complex that mediates degradation of β-catenin by the ubiquitin-proteosome pathway (53, 54). Initiation of the Wnt/wingless signal transduction pathway, which inhibits GSK 3β, or mutations in the β-catenin binding site on *APC*, both result in stabilization of cytosolic β-catenin, enabling it translocate into the nucleus, where it

interacts with a family of high mobility group-box (HMG) containing transcription factors (TCF-1, TCF-2, TCF-4, LEF-1) and initiates transcription of genes (45, 51, 52, 55-56). A few of the target genes have been found to be those involved in the cell cycle and tumor progression, including *c-Myc* (57), *cyclin D1* (58), *c-Jun* (59), *survinin* (60), *matrilysin* (61), *fibronectin* (62) and *PPARδ* (63).

Thus, the regulation of cytoplasmic levels of β-catenin is likely an important function of APC. This is further corroborated by the finding that gain-of-function mutations in the NH2 regulatory domain of β-catenin occurs in 48% of human colorectal tumors lacking *APC* mutations (64). However, important tumor suppressive properties of APC are also likely mediated through interactions with other cellular proteins. Some of these events involve apoptosis, cytoskeletal reorganization, chromosomal stability, and cell migration and mobility (reviewed in 50).

B.3.2. *K-ras.*

The *k-ras* gene, located on chromosome 12p encodes a 21-kDa, guaninine nucleotide binding protein with intrinsic GTPase activity (35). The *ras* proteins (k-*ras*, N-*ras*, and H-*ras*) are involved in transducing mitogenic signals from receptor tyrosine kinases in response to growth factors, cytokines, and hormones (35, 65). Binding of GTP activates *ras* whereas GTP hydrolysis, facilitated through GTPase activating proteins (GAPs), leads to the inactive, GDP-bound form (65). *Ras* oncogenes are made constitutively active through point mutations occurring at amino acids 12, 13, or 61, rendering them insensitive to GAPs (35, 65). Mutations in *k-ras* generally occur later in

adenoma growth and have been detected in 50% of colorectal carcinomas and in adenomas larger than 1 cm in size (35).

B.3.3 *DCC, DPC4(Smad4), and JV18-1(Smad2).*

One copy of chromosome 18q is lost in 47% of advanced adenomas and 73% of adenocarcinomas, but the target gene(s) has not been conclusively determined (11, 35). One candidate is deleted in colon cancer (*DCC*), which encodes a large transmembrane protein that contains immunoglobulin-like domains and shares homology with neural adhesion molecules (11, 35). The protein is expressed in a variety of tissues at points of cell-cell contact, suggesting a role in differentiation (66). Two other candidate genes include deleted in pancreatic carcinoma (*DPC4,* or *Smad4)* and *JV18-1* (also called *Smad2*) (35). Both of these proteins function downstream in the transforming growth factor (TGF)-β signaling pathway (11, 35). TGFβ exerts an inhibitory effect on cell growth in normal colorectal epithelium (67).

B.3.4 *p53.*

Mutational inactivation of the *p53* gene occurs late in tumor progression, most likely in the adenoma-carcinoma transition (9-10, 35). p53 normally functions to mediate anti-proliferative processes and/or apoptosis in response to cellular stresses (reviewed in 68, 69). Genomic damage, hypoxia, and aberrant oncogene expression stabilize p53, allowing transactivation of genes such as $p21^{WAF/CIP}$ (69-72) and Growth Arrest and DNA-Damage Induced (*GADD*) 45α (69-70, 73). These proteins block progression through the cell cycle and allow repair of DNA damage by inhibiting the activity of

cyclin dependent kinases (68, 69). p53 also mediates apoptosis in response to cellular damage through transcription-dependent and independent mechanisms, but the signaling pathways involved have not been completely elucidated (68, 69).

B.3.5 Epigenetic events.

In addition to the model proposed by Fearon and Vogelstein, aberrant methylation patterns are frequently observed in colorectal cancers. Methylation of 5'cytosine residues in gene-specific promoter regions is a naturally occurring modification of DNA that is heritable (74). Hypermethylation of certain promoter regions leads to transcriptional silencing whereas hypomethylation can lead to transcriptional activation. In both benign and malignant colon tumors global hypomethylation of DNA is frequently observed (75). In addition, promoter-specific hypermethylation in genes including the tumor suppressors *APC*, *p16*, and $p14^{ARF}$ and those involved in mismatch repair have been reported (74). These changes would likely contribute to cancer by affecting cell cycle regulation and capability of DNA repair, the latter leading to a "mutator" phenotype observed in a subset of tumors.

B.4. Inflammation/NSAIDS.

Although specific genetic mutations are required for initiation and propagation of tumorigenesis, modifier genes and/or the tumor microenvironment can also substantially contribute to colon cancer development. Inflammation has long been recognized to be involved in the etiology of CRC. Patients with a history of recurrent ulcerative colitis are at an increased risk of developing the disease (29). Additionally, Waddell *et al.* (76)

recognized over 25 years ago that administering the NSAID sulindac to patients with the inherited condition familial adenomatous polyposis (FAP) caused a significant regression of rectal polyps. Since then, several studies in humans (reviewed in 77) and animals (78-84) have established that NSAIDs inhibit colorectal tumorigenesis. In a review of published studies, the authors estimated a 40-50% reduction in sporadic colon adenomas, cancer, and mortality for regular users of NSAIDs (77).

The anti-neoplastic properties of NSAIDs have not been fully established. They bind to and inhibit the activity of cyclooxygenase (COX) enzymes, which catalyze the conversion of arachidonic acid to prostaglandins (PG) and thromboxane (TX) (85). The gastrointestinal tract contains two isoforms of COX. COX-1 is constitutively expressed and important for normal physiological processes such as maintenance of the gastrointestinal mucosa whereas COX-2 is normally expressed at very low levels but can be induced by pro-inflammatory cytokines, growth factors, lipopolysaccharides (LPS), and mitogens (85).

Treatment of cancer cells with NSAIDs increases apoptosis, induces cell-cycle arrest, and inhibits angiogenesis, presumably through down-regulating the synthesis of prostaglandins (85-89). Because increased levels of COX-2 have been detected in up to 90% of human colon cancer tissues (85), in adenomas from Min^{APC} mice (84), and in colon tumors from rodents induced with carcinogen (90), most of the anti-neoplastic effects of NSAIDs are thought to be mediated by this isoform. Boolbol *et al* (84) reported elevated COX-2 and PGE_2 in Min^{APC} mouse intestines compared wild-type littermates. Treatment of mice with sulindac inhibited tumor formation, reduced both COX-2 protein and PGE_2 levels, and restored enterocyte apoptosis. More direct evidence

for involvement of COX-2 in tumorigenesis was demonstrated by Oshima *et al* (91) who reported targeted disruption of COX-2 in $Apc^{\Delta 716}$ knockout mice reduced polyp formation by ~86% and also reduced polyp size (91). However, NSAIDs that are not selective for COX-2 such as aspirin and sulindac sulphone are also as effective in inducing apoptosis and suppressing tumorigenesis *in vivo,* suggesting that some of the anti-neoplastic effects of NSAIDs may be mediated through PG-independent pathways (92-94).

B.5. Carcinogen-induced model of colon cancer.

Although CRC was one of the first cancers to be characterized in terms of a genetic model, examining the etiology of carcinogenesis in humans is hampered due to the lack of specific prognostic biomarkers, heterogeneity among population groups, and the latency of time it takes for colorectal tumors to manifest. The use of animal models has proven a useful tool for studying various aspects of human disease under controlled laboratory conditions. Druckery and colleagues were the first to demonstrate that administration of the synthetic compound 1,2-dimethylhydrazine (DMH) or one of its metabolites azoxymethane (AOM) specificity and reliably induced colon tumors in rodent species (95-97). Following administration, DMH and AOM are metabolized in the liver to methyldiazonium (MDM) (97). MDM is an active methylating agent that forms a number of DNA adducts *in vivo,* with O^6-methyldeoxyguanosine (O^6-mdg) being one of the major mutagenic DNA lesions produced (98). Prior to replication, this lesion can be repaired in a "suicide" fashion by O^6-alkylguanine-DNA alkytransferases (O^6-atase) or removed by targeted apoptosis (98). The inability to remove O^6-mdg by either

of these processes can cause G:C to A:T transitions during DNA replication, a common genetic alteration reported in some human tumors (99).

The carcinogen-induced rodent model is one of the most widely used models for studying colon carcinogenesis. Tumors develop almost exclusively in the colon, primarily in the distal region, similar to the distribution observed in humans from high-risk areas. Although *APC* mutations are rare in carcinogen-induced rodent colon tumors, mutations in the GSK-3β phosphorylation consensus sites on β-catenin have been reported (100). These sites are important for down-regulation of β-catenin by ubiquitination and likely affect the stability of the protein. Takahashi *et al.* (100) reported that in normal colon epithelium, β-catenin was primarily localized to the plasma membrane, whereas a majority of dysplastic ACF, adenomas, and adenomacarcinomas exhibited homogenous cytoplasmic and scattered nuclear staining. k-*ras* mutations on codon 12 and 13 in DMH-induced colon adenocarcinomas have also been reported to occur in a similar frequency to that observed in humans (101-102). In addition, local inflammation, promoted in part through up-regulation of COX-2 and inducible nitric oxide synthase (iNOS), is another common characteristic involved in the pathogenesis of colon cancer from both species (103-104).

C. DIETARY FACTORS AND COLORECTAL CANCER.

Dietary habits are strongly linked to the etiology of CRC (2-4). Numerous epidemiological studies have been conducted in attempts to identify specific dietary components associated with higher risk of developing CRC or contributing to CRC mortality (reviewed in 2-4, 105). Generally, data are most consistent for a lower occurrence of CRC in individuals consuming higher intakes of vegetables, particularly

raw, green, and cruciferous vegetables, and dietary fiber. Dietary components associated with an increased risk include red meat and alcohol, and possibly dietary fat, processed and heavily cooked meats, and sucrose (2, 4, 105). Metabolic consequences related to over-nutrition, including obesity and insulin resistance have also been implicated in the etiology of CRC (22-23, 106-112). Several hypotheses have been generated to explain the association of diet and lifestyle habits with CRC and further tested in animal models. There is currently no consensus as to how diet consistently modulates risk, which is undoubtedly multi-factorial and likely depends on the ratio of both promoting and anti-promoting factors. Some of the more established mechanisms are presented in **TABLE 1** and are discussed below.

Red and processed meats may increase CRC by increasing lumenal concentrations of potentially carcinogenic compounds (113-114). Heterocyclic amines (HCA), produced by cooking meat at high temperatures are mutagenic in bacterial assays (115) and dose-dependently induce large intestinal tumors when administered to rodents (116-117). Meat consumption has also been associated with higher intakes of dietary fat. Dietary fat and cholesterol stimulate bile acid secretion and populations at increased risk for colon cancer excrete higher levels of bile acids in the stool (118). Approximately 2-5% of bile acids escape reabsorption in the small intestine and are metabolized by colonic microflora producing the secondary bile acids deoxycholate and litholicholic acid (119). Secondary bile acids are irritating to the epithelium (120), induce cell proliferation (120), and have been demonstrated to promote experimental colon cancer when administered to animals (121-123). Adequate intakes of dietary calcium can form insoluble salts with lumenal bile and fatty acids and calcium supplementation reduces hyperproliferation in

TABLE 1. Proposed mechanisms relating diet and nutrition-related factors to CRC risk.

	Risk Factors	Potential mechanisms	References
Increased Risk	Red & processed meat	• Increases lumenal concentration of potentially carcinogenic *N*-nitroso compounds and heterocyclic amines	113-115
	Alcohol consumption	• Increases mucosal proliferation • Initiation of DNA-acetaldehyde adducts • May affect DNA repair	130-132
	Inadequate folate/methionine	• May affect DNA methylation status and gene transcription • Low folate decreases synthesis of dTMP from dUMP, which can cause point mutations if not repaired.	136-141
	High dietary fat	• Increases lumenal bile acids, which act as irritants to the mucosa	118, 120-123
	Obesity & Insulin Resistance	• Direct effects of insulin on mitogenesis of colon cells • Increases intravascular energy • Increases bioavailability of IGF-1 growth hormone	reviewed in 211
Decreased Risk	Vegetables	• Fiber (see below) • Antioxidants – reduce oxidative damage • Induces phase II detoxification enzymes, reduces inflammation/cancer cell growth	152-156, 164-178
	Non Starch Polysaccharides/ Dietary Fiber	• Non-fermentable fibers speed intestinal transit, may dilute fecal contents/irritants • Fermentable fibers lower lumenal pH, which can inhibit formation of secondary bile acids. Bacterial fermentation produces the SCFA acetate, propionate and butyrate. Butyrate inhibits cancer cell growth and is pro-apoptotic *in vitro*.	180, 183-185
	Calcium	• Precipitates bile acids and reduces irritation/hyperproliferation	124-129
	n-3 fatty acids	• Reduces proliferation, increases differentiation and apoptosis • Alters membrane phospholipid composition, reduces PGE_2 and TX_{B2} • Decreases activation of $p21^{ras}$, PKCβII, COX-2	200, 202, 208-210

the colorectal mucosa of rodents (124-125) and in humans at increased risk for CRC (126-129).

Moderate alcohol consumption potentially increases CRC risk by influencing mucosal cell proliferation (130-131), inhibiting DNA repair (132), and/or through generation of DNA-acetylaldehyde adducts (132). The effect of alcohol on CRC risk appears to be potentiated by insufficient dietary intake of folate or methionine (133-134). Folate is required for normal functioning of the methionine cycle. Tetrahydrofolate (THF) is the reduced form of folic acid capable of accepting one-carbon groups from metabolites generated in amino acid metabolism and serves as a precursor for 5 coenzyme forms (135). These THF derivatives participate in distinct one-carbon metabolism pathways including amino acid metabolism and purine and pyrimidine synthesis (135-137). N^5-methyltetrahydrofolate (N^5-methyl THF) regenerates methionine from homocysteine, which can be converted to S-adenosyl-methionine (SAM). SAM is required for biological methylation reactions, and may be reduced when diets are deficient in labile methyl groups (137). This can result in DNA hypomethylation, which may enhance oncogene expression (138). Aberrations in 5,10 methylene tetrahydrofolate (a metabolite of folate) may also result in misincorporation of dUMP for dTMP during DNA replication, thereby enhancing point mutations (136-137). In rapidly dividing cells such as the intestinal epithelium, enzymatic mechanisms may not sufficiently repair DNA damage prior to proliferation, causing heritable changes to be "fixed". In support of this, diets deficient in folate have been demonstrated to produce DNA strand breaks in the p53 gene in rat colon mucosa (139) and were associated with higher prevalence of k-*ras* mutations in human colon adenomas (140-141). However, these diets contained sub-

optimal levels of dietary folate and it is not likely that intakes exceeding current recommendations would provide any additional benefit to CRC.

Vegetables contain several antioxidant vitamins and minerals as well as bioactive compounds that may contribute to reduced CRC risk (2, 142-151). Antioxidants may inhibit carcinogenesis through scavenging reactive oxygen species (ROS) and preventing ROS-induced DNA damage (152-156). Epidemiological and experimental data however do not indicate that supplementation of antioxidant compounds beyond recommendations is associated with reduced occurrence of CRC or colorectal polyps (157-163). Several bioactive compounds present in vegetables have been suggested to exert anti-cancer properties through a variety of mechanisms including induction of hepatic and intestinal phase II detoxification enzymes (164-168), modulating oncogene activation (169-171), and anti-inflammatory properties (172-176), among others (177-178). Vegetables and cereal grains are also rich sources of dietary fiber, which is related to lower colon cancer rates in some epidemiological studies (2, 179). However, dietary fibers differ in physiochemical characteristics and identifying mechanisms by which fiber consistently modulates colon carcinogenesis is difficult. Less fermentable fiber constituents and sources including cellulose, lignins, and wheat bran may affect colon cancer by speeding intestinal transit and diluting and adsorbing lumenal carcinogens or irritants. Bacterial fermentation of more soluble fibers and non-starch polysaccharides lowers lumenal pH, which can affect microbial metabolism and generation of secondary bile acids (180). Fermentation also produces the short-chain fatty acids (SCFA) acetate, propionate, and butyrate (181-182). Butyrate has been demonstrated to inhibit colon cancer cell growth and induce apoptosis *in vitro* (183-185). Numerous animal studies have been conducted

to examine the influence of purified fiber sources on experimental colon carcinogenesis and inconsistent results have been reported (186-198). Generally, wheat bran protects against colon cancer more consistently than cellulose or more fermentable fiber sources (187, 190-191, 194, 198). These results suggest that purified fibers alone do not appear to inhibit tumorigenesis, whereas it is likely that some other factors present in vegetables and grains and/or synergistic effects of fiber with other dietary components may be responsible for anti-cancer properties.

Limited data are available for n-3 fatty acid consumption and CRC risk, although animal studies have consistently shown a protective effect of fish oil on experimental colon carcinogenesis (199-204). The inhibitory effects of fish oil on colon cancer have been associated with enhancing cellular differentiation and apoptosis (200, 202), suppressing the expression and/or activation of $p21^{ras}$ and COX-2 (205-207), reducing protein kinase C (PKC) βII activity (208), and altering synthesis of prostaglandins (209-210) and TX_{B2} (210) in non-neoplastic mucosa.

Aside from specific dietary components, metabolic disorders related to over-nutrition have been proposed to contribute to higher incidence of CRC. McKeown-Eyssen (106) and Giovannucci (107) were the first to recognize that several risk factors associated with CRC, such as sedentary lifestyles, obesity, and high fat diets were similar to those for insulin resistance and diabetes. This led to the proposal that metabolic consequences associated with insulin resistance (hyperinsulinemia, hyperglycemia, hyper-triglyceridemia, increased non-esterified fatty acids (NEFAs)) promotes colon carcinogenesis (106-107). Several possibilities for this association have been suggested. First, high circulating levels of insulin may directly promote colonic tumorigenesis

directly through receptor binding and mitogenesis. Second, insulin resistance may indirectly affect colon epithelial proliferation through increased intravascular energy provision. A third model suggests that hyperinsulinemia may lead to increased insulin-like growth factor-1 (IGF-1) bioavailability, a mediator of cell survival and growth, by reducing hepatic IGF binding protein 1 (IGFBP1) production (211).

The epidemiology studies relating insulin resistance to colon cancer have been recently reviewed (109). Most case-control and cross-sectional studies show a slightly elevated risk of CRC in diabetics, with odds ratios (OR) ranging from 1.0-2.9 (109). Similar results have been reported using cohorts of patients with diabetes. However, some of these studies are limited in interpretation because most relied on self-reporting of diabetes and were not designed to address the extent of glycemic control. Prospective studies evaluating biomarkers of insulin-resistance (*i.e.* C-peptide, HgB_{1C}) rather than history of diabetes generally show stronger positive associations, particularly for advanced CRC and CRC mortality (109, 111-112). Increased risk for CRC has also been reported in individuals with elevated plasma IGF-1 (112, 212) and in individuals with acromegly, who have pathologic elevations of serum growth hormone and IGF-1 (pooled OR=2.04) (213).

Colon cancer cells express both insulin (214) and IGF-1 receptors (IGF-1R) (215-216), and treatment of cells with either insulin or IGF-1 stimulates mitogenesis (217-218). Additionally, IGF-1 up-regulates the expression of vascular endothelial growth factor (VEGF) (219) and cell lines over-expressing IGF-1R are resistant to apoptosis and are more metastatic than non-transfected cells (220). In animal studies, repeated administration of insulin significantly increased the multiplicity of ACF (221) and the

number of colon tumors (222) in rats chemically induced with AOM, suggesting a direct effect of insulin on tumor promotion. Koohestani *et al.* (223) conducted a study to compare the effect of diet on insulin resistance and colon carcinogenesis and found that high energy, high fat diets led to impaired glucose tolerance, which was positively correlated with the size of ACF ($r = 0.67$, $p<0.001$).

D. LEGUMES AND COLORECTAL CANCER.

Legumes (peas, beans, lentils, peanuts) are one dietary component traditionally consumed in populations where cancers of the colon, breast, and prostate are low (7), and several of the mechanisms proposed to reduce CRC risk in humans and animals can be achieved through increased consumption of legumes (**TABLE 1**). Legumes are unique plant foods, providing a concentrated source of vegetable protein and a variety of both essential and non-essential nutrients (224-226) (**TABLE 2**). Oilseeds, including soybeans, and peanuts are higher in lipid content (24%) than dry beans (1-2%), but both are a significant source of complex carbohydrates and both soluble and insoluble dietary fiber. Legumes are a good source of the minerals iron, calcium, copper, zinc, potassium, and magnesium and the water-soluble vitamins thiamine, niacin, riboflavin and folate (224-226). Several bioactive compounds including saponins, phytic acid, phenolics, tannins, phytosterols, lectins and protease inhibitors are also present in legumes and some have been shown to exert a number of health benefits (224-227).

In the US, soybeans are typically processed prior to consumption to generate products with a higher protein content and to extract oil for commercial use (228). Typically, soy products are grouped into three categories based on their protein content:

TABLE 2. Nutrient composition of common beans and soy products per 100 g[a]

	Energy (kcal)	g Carbohydrate minus fiber[b] (% dry wt)	g Protein (% dry wt)	g Fat (% dry wt)	g Fiber (% dry wt)	µg Folate	mg Calcium	mg Iron
Dry (common) beans								
Black bean	132	24 (44%)	8.9 (26%)	0.54 (2%)	8.7 (25%)	149	27	2.1
Pinto bean	143	26 (46%)	9.0 (24%)	0.65 (2.0%)	9.0 (24%)	172	46	2.1
Kidney bean	127	23 (50%)	8.7 (26%)	0.50 (2%)	6.4 (19%)	130	35	2.2
Navy bean	140	26 (43%)	8.2 (23%)	0.62 (1.7%)	11 (25%)	140	69	2.4
Soybeans (oil seeds) and soy products								
Soybean	173	10 (11%)	17 (44%)	9.0 (24%)	6.0 (16%)	54	102	5.1
Defatted soy flour	330	38 (23%)	47 (51%)	1.2 (1%)	18 (19%)	305	241	9.2
Soy concentrate (alcohol washed)	331	31 (27%)	58 (62%)	0.46 (1%)	5.5 (6%)	340	363	10.8
Soy protein isolate (potassium type)	338	2.6 (1%)	88 (93%)	0.53 (1%)	2.1 (2%)	176	178	14.5
Tofu (Mori-Nu, silken, extra firm)	55	2.0 (16%)	7.4 (62%)	1.9 (16%)	0.10 (0.8%)	--	31	1.2

[a] Values obtained from USDA Standard reference, 15 (230) for mature, boiled seeds (without salt).
[b] Total carbohydrate (starch, sugars, oligosaccharides) minus dietary fiber.

soy flour, soy concentrates, and soy isolates. All three products are derived from defatted soy flakes and range in crude protein content from 50% (dry wt) in defatted soy flour to >90% protein in soy isolates (**TABLE 2**). Soy flours and soy grits are the least processed of the three and are made by grinding and screening soybean flakes (229). Soy concentrates are prepared through aqueous or aqueous-ethanol extraction of defatted soy flakes to remove soluble carbohydrate fractions. Isolates are the most refined soy protein source through which most of the total carbohydrates from soy flour are removed. Soy protein products are used in a variety of second-generation soy foods, bakery products, breakfast cereals, and infant formulas, accounting for more than 90% of soybeans consumed in the United States (228).

There has been renewed interest in studying legumes with respect to chronic disease prevention (225-227, 231-232). Several epidemiological and experimental studies have implicated that incorporating more legumes into the diet has the potential to aid in the prevention and/or management of several chronic diseases including diabetes (225, 231-236), cardiovascular disease (6, 225-227, 231-232, 237-241), obesity (231-232, 242-244), and cancer (225, 227, 5-7). These diseases are more common in developed countries, such as the United States, where consumption of beans and other plant products remains below recommendations (225).

D.1 Epidemiological Studies.

Most epidemiological studies examining relationships between diet and cancer put little emphasis on legume/pulse consumption. In studies where legume intake is assessed, distinctions are usually not made between the various legumes, making direct correlations with colon cancer difficult. In two prospective studies examining dietary

patterns and disease risk as part of the Adventist Health Study in the US, significant inverse associations between legume consumption and colon cancer were reported (5-6). In the study by Singh and Fraser (5), individuals consuming legumes > 2 times/week were 47% less likely to develop colon cancer when compared to individuals consuming legumes never to < 1 time/week. Two case-control studies, one conducted in Australia and the other in the Spanish island of Majorca, also reported a protective effect of legume consumption on colon cancer risk. In the study by Steinmetz and Potter (245), the OR was 0.4 and 0.7 for women and men with the highest legume (beans, split peas, lentils, soybeans, chickpeas) intakes, respectively. Another case-control study in Majorca reported on fiber from pulses, rather than as a group. Similar to the previous studies, the authors reported a significant protective effect ($P < 0.01$) for individuals in the highest quartile of legume fiber intake, with an OR of 0.4 (246). Correa (7) was the only study to specifically examine bean consumption. Per capita data compiled from 41 countries, revealed that countries with the greatest consumption of beans had the lowest mortality rates due to breast ($r = -0.70$), prostate ($r = -0.66$), and colon ($r = -0.68$) cancer (7).

The epidemiological studies addressing soy intake and colorectal cancer risk have been recently reviewed (247-248). A total of 13 studies (3 ecological, 1 cohort, and 9 case-control) have been conducted. In the three ecological studies examining soy intake and mortality from colon or rectal cancer, 1 found a significant inverse association (249), one found a positive association (250), and the third reported no significant association (251). Among the remaining studies, the risk ratios reported for soy consumption and colon or rectal cancer ranged from 0.48 to 1.9 (252-261). Only two studies found a significant inverse association between soy consumption and colon cancer after

adjustment for multiple confounding variables. The first reported a 50% lower risk of colon cancer only in women consuming higher soy products (260) and the other found a 50% reduction in risk of colorectal polyps with increased consumption of tofu or soybeans (259). The interpretation of results from the remainder of the studies is limited due to the incomplete assessment of soy intake and because most studies did not adjust for confounding variables (248). However, despite these limitations, there was some suggestion of an inverse association between soy consumption and colon cancer and between non-fermented soy products and rectal cancer (248).

One clinical study was conducted to determine if consumption of soy protein isolate could reduce early biomarkers associated with colon cancer development (262). Subjects with a history of colon cancer or polyps consumed 38 g of soy protein isolate containing 70 mg of isoflavones or 38 g of casein for one year. Biopsies of colon tissue were taken at the start of the study and after one year of intervention. The author reported a downward shift in the proliferation zone in colonic crypts obtained from individuals consuming the soy protein isolate, suggesting soy consumption reduces colon cancer risk by enhancing cellular differentiation (262).

D.2 Experimental studies.

Only two studies have examined the potential of dry beans to inhibit colon cancer. Hughes *et al.* (14) fed rats diets containing either pinto beans or casein as the protein source and found that feeding pinto beans inhibited colon cancer by 50% and significantly reduced the number of tumors that developed. In another study, Hangen and Bennink (15) reported that feeding either black beans or navy beans inhibited colon

cancer by ~57%, and similar to Hughes *et al.* (14), bean-fed rats also developed fewer tumors. In this study, the chemoprevention of beans was associated with significantly more resistant starch reaching the colon, higher colonic acetate and butyrate production, and a decrease in body fat (15).

The experimental data examining the potential of soy-based diets to inhibit chemically induced colon cancer in rodents is inconsistent. This is due, in part to differences in experimental protocols, the type of soy products (soy flour/meal or isolated soy proteins) utilized, and end-point biomarkers examined. Processing of soybeans can affect the content of carbohydrates, fiber, and various bioactive compounds. Because some of these compounds have been reported to have anti-carcinogenic properties, it is likely that consumption of soy products that are minimally processed may be more beneficial in reducing risk of colon cancer.

Experiments where soy flour was examined and feeding began following carcinogen administration (promotion), a protective effect on both preneoplastic lesions and colon cancer has been reported (16-18, 263). In a series of studies, Bennink *et al.* (16-18) compared the potential of diets containing soy flour (full-fat or defatted), casein, or ethanol-washed soy concentrate to inhibit colonic tumor development during the promotional phase of carcinogenesis. They consistently reported a reduction in colon tumor incidence and tumor burden in rats fed defatted soy flour (43%, 0.67 tumors/animal) compared to rats fed either soy concentrate (68%, 1.1 tumor/animal) or casein (68%, 1.43 tumor/animal). However, two animal studies that fed rats either soy flour or soybean meal both prior to and after carcinogen administration have produced inconsistent results (264-265). One found an increase in colon tumors (264) in soybean

meal-fed animals ($P = 0.15$) whereas the other study found a significant reduction in tumor incidence in animals fed defatted soy flour ($P < 0.05$) (265).

Five experimental studies have examined the influence of soy protein isolates high or low in isoflavones on colon carcinogenesis (44, 266-269). With the exception of one study (269), isolates do not appear to influence colon tumorigenesis (44), and may enhance the development of ACF when fed prior to carcinogen injection (44, 267-268), although this does not appear to correlate with increased tumorigenesis (44). These studies indicate that minimally processed soy products more consistently inhibit colon cancer than either soy concentrates or isolates and chemoprevention appears associated with one or more bioactive compounds present in soy.

D.3 Non-nutritive bioactive compounds.

The inverse relationship between consumption of plant-based foods and lowered risk of several cancers has generated interest in identifying compounds with anti-cancer potential. Legumes contain several compounds including saponins, protease (Bowman-Birk) inhibitors, phenolics, phytoestrogens, and phytic acid that have been examined with respect to colon cancer inhibition. In an attempt to summarize these studies, protease inhibitors (270-272) and phytic acid (273-276) inhibit colon cancer most consistently in animal models, whereas phytoestrogens (17, 277-279) do not appear to modulate colon cancer risk. Limited data are available for saponins (280) and phytosterols (281).

The Bowman-Birk protease inhibitor (BBI) and phytic acid (IP_6) have been the most extensively studied components in legumes, and both consistently inhibit experimental carcinogenesis in the colon (270-276) and at other sites (282-289), The mechanisms of inhibition by either compound are not known with certainty. BBIs are

small (6-10 kDa) proteins that can bind to and inhibit both chymotrypsin and trypsin (143). BBI distributes among most tissues within 3 hours following oral administration, the highest concentrations remaining in intestinal contents and urine (290). Because the catalytic activity within tissues remains intact (291), Kennedy proposed that BBI may be inhibiting one or more enzymes involved in inducing the transformed phenotype (148-149, 291). In support of this, BBI has been associated with reversing the up-regulation of proteolytic activity in the oral epithelium following carcinogen treatment (292) and suppressing radiation-induced expression of proto-oncogenes (*c-myc*) in colon tissue and cancer cell lines (170, 293).

Vucinek and Shamsuddin (294) suggested that phytic acid (IP_6) inhibits tumor growth by affecting cellular signaling through lower inositol derivatives. There is some evidence that IP_6 interacts with the Akt-NFκB cell survival pathway (295). Sandra *et al.* (295) reported that pre-treatment of IP_6 to HeLa cells inhibited insulin or TNF induced Akt phosphorylation and NFκB translocation into the nucleus. In this study, neither PI-3 kinase activity nor the MAP kinase signaling pathway was affected by IP_6, however there was a significant reduction of Akt translocation to the plasma membrane, potentially through antagonist binding of IP_4 to the pleckstrin homology domain of Akt (295). Treatment of cells with IP_6 induces a G1 cell cycle arrest and reduces cells in the S phase in breast (296), colon (296), and prostate cancer cell lines (297). Additionally, IP_6 and inositol derivatives containing at least 3 phosphates (IP_5, IP_4, and IP_3) can bind divalent metals, and reduces iron catalyzed lipid peroxidation (298) and high iron-induced promotion of colon tumorigenesis in rats (299).

Most of the experimental evidence suggesting phytoestrogens, in particular genistein, may prevent cancer has been derived from *in vitro* studies. Genistein suppresses growth and induces apoptosis in a variety of cancer cell lines (300-308). Genistein has also been shown to inhibit lipid oxidation (309-310), suppress angiogenesis (311-314), and at high concentrations inhibits tyrosine kinases (315). However, animal studies examining the potential of purified phytoestrogens to modulate colon carcinogenesis are generally not supportive of a protective effect (17, 277-279). Four studies have been conducted long enough for tumors to develop and neither found a significant reduction in colon tumorigenesis by feeding genistein (277, 279), genistin (17), or a mixture of soy isoflavones (17, 280-281). In one study, genistein (250 μg/g diet) enhanced tumor multiplicity in the colon of rats administered AOM (277). Similarly, Bennink *et al.* (17) fed genistin or a mixture of isoflavones (genistin, daidzin, and glycitin) in amounts comparable to that in defatted soy flour and found that genistin increased the number of rats with tumors ($P = 0.06$), although the tumors that developed were smaller than in controls. The other two studies found no effect of phytoestrogens on intestinal tumorigenesis (278-279). Therefore, phytoestrogens fed as purified compounds are likely not sufficient to inhibit colon cancer in established animal models.

E. RATIONALE.

CRC is a significant health problem in industrialized countries. In the United States, it is the fourth most common cancer and the second leading cause of cancer mortality (19). Despite improvements in adjunct therapy for those diagnosed with colorectal cancer, the relative five-year survival rate is currently only 63% (19). When detected at an early, localized stage, the overall prognosis improves. However, due to low rates of screening in the general population, only 39% of CRC cases are diagnosed at this early stage (19). Clearly, prevention strategies aimed at early detection and modifying risk factors have the potential to reduce medical costs and age-adjusted mortality associated with the disease.

Dietary habits strongly influence the development of CRC, and dietary factors associated with an increased or decreased risk for CRC have been identified (2-4) **(TABLE 1)**. Several of the mechanisms proposed to explain the relationship of diet to risk of colon tumorigenesis are related to modulation of mucosal abnormalities that favor adenoma formation. For example, in the colon of individuals at high risk for CRC and in animals treated with colon carcinogens, an upward shift of proliferative epithelial cells towards the lumen has been described (37, 40). The anti-cancer properties of some dietary components have been attributed to reversing mucosal hyperproliferation (124-129, 262), either through enhancing apoptotic indices (200, 202), favoring differentiation (200, 202, 262), and/or modulating mitogenic enzyme activity (201, 205-208).

Recent studies have begun to explore dietary differences in mucosal gene expression utilizing microarrays, and have revealed a more complex involvement of diet on expression of genes involved in apoptosis, immunity, and endocrine changes

potentially contributing to colon cancer inhibition (316-317). Because the anti-neoplastic effects of many dietary components precede adenoma formation, further characterization of these events may lead to a more thorough understanding of diet on CRC etiology and provide a rationale basis for specific public health recommendations aimed at colon cancer prevention.

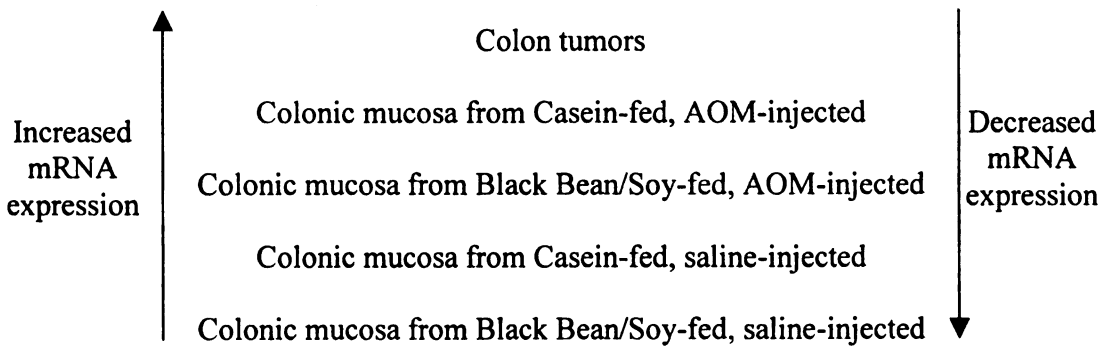
The AOM-induced rodent model is frequently utilized to examine dietary influences on colon carcinogenesis. Using this model, dry beans (14-15) and soy flour (16-18) have been previously demonstrated to inhibit the both the incidence and multiplicity of colonic tumors compared to rats fed a control (casein-based) diet. The mechanisms underlying altered susceptibility to colon cancer by beans have not been fully elucidated. Because beans inhibit the occurrence and the number of tumors (14-18) that develop rather than the size of colon tumors (mg tumor/tumor bearing rat) (15), the current study was designed to examine potential chemopreventative mechanisms of beans early in the neoplastic process. Therefore, the **central hypothesis** of this research is that beans inhibit colon cancer by modulating mucosal expression of genes regulating epithelial cell growth and differentiation. The **primary objectives** were to: 1) identify genes involved in cellular growth and differentiation that are significantly increased or decreased in colon tumors compared to normal colonic epithelium; and 2) identify which of these gene changes in non-neoplastic mucosa were attenuated by feeding beans using microarrays.

To test my hypothesis, three experiments were conducted:

1. In the first experiment, microarrays were used to profile genes differentially

expressed in rat AOM-induced colon cancers compared to non-neoplastic colonic mucosa. Because AOM is a global methylating agent and early gene changes have not been previously described, results from this experiment provided insight into biological processes involved in tumor promotion **(CHAPTER III).**

2. Having determined genetic alterations associated with cancer promotion, the second experiment was conducted to profile genes altered in non-neoplastic mucosa to identify early cellular events permissive for tumorigenesis and those affected by dietary treatment. Microarrays were performed on RNA isolated from normal-appearing colonic mucosa in rats treated with either carcinogen (AOM) or saline and fed a control (casein)-, black bean-, or soy-flour based diet. I anticipated that genes important in dietary suppression of colon carcinogenesis would have altered expression (increased or decreased) that correlated with tumor incidence and proceeded in the following sequence **(CHAPTER IV).**



3. The third experiment was conducted to determine if diet-related differences in gene changes correspond to alterations in epithelial kinetics and colonic inflammation using immunohistochemical techniques **(CHAPTER V).**

F. LITERATURE CITED.

1. Ferlay J, Bray F, Pisani P and Parkin DM. (2002) *GLOBOCAN 2002: Cancer incidence, mortality, and prevalence worldwide.* IARC Press, Lyon, France, http://www-depdb.iarc.fr/globocan/GLOBOframe.htm.

2. American Institute of Cancer Research/World Cancer Research Fund (AICR/WCRF). (1997) Colon, rectum. In *Food, Nutrition and the Prevention of Cancer: A Global Perspective.* BANTA Book Group, Menasha, WI, pp. 216-51.

3. Potter JD, Slattery ML, Bostick RM and Gapstur SM. (1993) Colon-Cancer - a Review of the Epidemiology. *Epidemiologic Reviews.* 15(2): 499-545.

4. Potter JD. (1999) Colorectal cancer: Molecules and populations. *Journal of the National Cancer Institute.* 91(11): 916-932.

5. Singh PN and Fraser GE. (1998) Dietary risk factors for colon cancer in a low-risk population. *American Journal of Epidemiology.* 148: 761-774

6. Fraser GE. (1999) Associations between diet and cancer, ischemic heart disease, and all-cause mortality in non-Hispanic white California Seventh-day Adventists. *American Journal of Clinical Nutrition.* 70: 532S-538S.

7. Correa P. (1981) Epidemiological correlations between diet and cancer frequency. *Cancer Research.* 41: 3685-3689

8. Vogelstein B, Fearon ER, Hamilton SR *et al.* (1988) Genetic Alterations During Colorectal-Tumor Development. *New England Journal of Medicine.* 319(9): 525-532.

9. Fearon ER and Vogelstein B. (1990) A Genetic Model for Colorectal Tumorigenesis. *Cell.* 61(5): 759-767.

10. Kinzler KW and Vogelstein B. (1996) Lessons from hereditary colorectal cancer. *Cell.* 87(2): 159-170.

11. Ilyas M, Straub J, Tomlinson IPM and Bodmer WF. (1999) Genetic pathways in colorectal and other cancers. *European Journal of Cancer.* 35(3): 335-351.

12. Grady WM and Markowitz SD. (2002) Genetic and epigenetic alterations in colon cancer. *Annual Review of Genomics and Human Genetics.* 3: 101-128.

13. Pories SE, Ramchurren N, Summerhayes I and Steele, G. (1993) Animal models for colon carcinogenesis. *Archives of Surgery.* 128: 647-53.

14. Hughes JS, Ganthavorn C and Wilson-Sanders S. (1997) Dry beans inhibit azoxymethane-induced colon carcinogenesis in F344 rats. *Journal of Nutrition.* 127: 2328-2333

15. Hangen LA and Bennink MR. (2003) Consumption of black beans and navy beans (Phaseolus vulgaris) reduced axozymethane-induced colon cancer in rats. *Nutrition and Cancer.* 44: *60-6*

16. Bennink MR and Om AS. (1998) Inhibition of colon cancer (CC) by soy phytochemicals but not by soy protein. *Faseb Journal.* 12(5): 3808.

17. Bennink MR, Om AS and Miyagi Y. (1999) Inhibition of colon cancer (CC) by soy flour but not by genistin or a mixture of isoflavones. *Faseb Journal.* 13(4): A50-A50.

18. Bennink MR, Om AS and Miyagi Y. (2000) Inhibition of colon cancer (CC) by wheat bran, soy flour, and flax. *Faseb Journal.* 14(4): A217-A217.

19. American Cancer Society. (2005) Cancer Facts & Figures 2005. http://www.cancer.org/downloads/STT/CAFF2005f4PWSecured.pdf.

20. Troisi RJ, Freedman AN and Devesa SS. (1999) Incidence of colorectal carcinoma in the US - An update of trends by gender, race, age, subsite, and stage, 1975-1994. *Cancer.* 85(8): 1670-1676.

21. Calvert PM and Frucht H. (2002) The genetics of colorectal cancer. *Annals of Internal Medicine.* 137(7): 603-612.

22. Bianchini F, Kaaks R, and Vainio H. (2002) Overweight, obesity, and cancer risk. *Lancet Oncology.* 3(9): 565-574.

23. Calle EE, Rodriguez C, Walker-Thurmond K and Thun MJ. (2003) Overweight, obesity, and mortality from cancer in a prospectively studied cohort of US adults. *New England Journal of Medicine.* 348(17): 1625-1638.

24. Calle EE and Thun MJ. (2004) Obesity and cancer. *Oncogene.* 23(38): 6365-6378.

25. Jee SH, Kim HJ, and Lee J. (2005) Obesity, insulin resistance and cancer risk. *Yonsei Medical Journal.* 46(4): 449-455.

26. Komninou D, Ayonote A, Richie JP and Rigas B. (2003) Insulin resistance and its contribution to colon carcinogenesis. *Experimental Biology and Medicine.* 228(4): 396-405.

27. Giovannucci E. (2003) Nutrition, insulin, insulin-like growth factors and cancer. *Hormone and Metabolic Research.* 35(11-12): 694-704.

28. Chang CK and Ulrich CM. (2003) Hyperinsulinaemia and hyperglycaemia: possible risk factors of colorectal cancer among diabetic patients. *Diabetologia.* 46(5): 595-607.

29. Eaden J. (2004) Review article: colorectal carcinoma and inflammatory bowel disease. *Alimentary Pharmacology & Therapeutics.* 20: 24-30.

30. Samad AKA, Taylor RS, Marshall T and Chapman MAS. (2005) A meta-analysis of the association of physical activity with reduced risk of colorectal cancer. *Colorectal Disease.* 7(3): 204-213.

31. Herendeen JM and Lindley C. (2003) Use of NSAIDs for the chemoprevention of colorectal cancer. *Annals of Pharmacotherapy.* 37(11): 1664-1674.

32. Grodstein F, Newcomb PA and Stampfer MJ. (1999) Postmenopausal hormone therapy and the risk of colorectal cancer: A review and meta-analysis, *American Journal of Medicine.* 106: 574-82.

33. Cummings JH and Macfarlane GT. (1991) The control and consequences of bacterial fermentation in the human colon. *Journal of Applied Bacteriology.* 70: 443-59.

34. Eastwood GL. (1983) Colon structure. In *Colon, structure and function,* (Bustos-Fernandez L., ed.), Plenum Medical Book Company, New York, pp. 1-16.

35. Kinzler KW and Vogelstein B. (1998) Colorectal tumors. In *The genetic basis of human cancer,* (Vogelstein B and Kinzler KW, eds.), McGraw Hill, New York, pp. 565-82.

36. Karam SM. (1999) Lineage commitment and maturation of epithelial cells in the gut. *Frontiers in Bioscience.* 4: d286-98.

37. Lipkin M. (1974) Phase I and phase II proliferative lesions of colonic epithelial cells in diseases leading to colonic cancer. *Cancer.* 34: 878-888.

38. Muto T, Bussey JR and Morson BC. (1975) The evolution of cancer of the colon and rectum. *Cancer.* 36: 2251-70.

39. Fenoglio CM and Lane N. (1974) The anatomical precursor of colorectal carcinoma. *Cancer.* 34: 819-23.

40. Winawer SJ, Zauber AG, Steward E *et al.* (1991) The natural history of colorectal cancer: opportunities for intervention. *Cancer.* 67: 1143-49.

41. Bird RP. (1995) Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. *Cancer Letters.* 93: 55-71.

42. Siu IM, Pretlow TG, Amini SB and Pretlow TP. (1997) Identification of dysplasia in human colonic aberrant crypt foci. *American Journal of Pathology.* 150(5): 1805-1813.

43. Hardman WE, Cameron IL, Heitman DW and Contreras E. (1991) Demonstration of the Need for End-Point Validation of Putative Biomarkers - Failure of Aberrant Crypt Foci to Predict Colon Cancer Incidence. *Cancer Research.* 51(23): 6388-6392.

44. Davies MJ, Bowey EA, Adlercreutz H *et al.* (1999) Effects of soy or rye supplementation of high-fat diets on colon tumour development in azoxymethane-treated rats. *Carcinogenesis.* 20(6): 927-931.

45. Oving IM and Clevers HC. (2002) Molecular causes of colon cancer. *European Journal of Clinical Investigation.* 32(6): 448-457.

46. Vogelstein B and Kinzler KW. (1993) The Multistep Nature of Cancer. *Trends in Genetics.* 9(4): 138-141.

47. Powell SM, Zilz N, Beazer Barclay Y *et al.* (1992) APC mutations occur early during colorectal tumorigenesis. *Nature.* 359: 235-37.

48. Miyaki M, Konishi M, Kikuchiyanoshita R *et al.* (1994) Characteristics of Somatic Mutation of the Adenomatous Polyposis-Coli Gene in Colorectal Tumors. *Cancer Research.* 54(11): 3011-3020.

49. Polakis P. (1997) The adenomatous polyposis coli (APC) tumor suppressor. *Biochimica Et Biophysica Acta-Reviews on Cancer.* 1332(3): F127-F147.

50. van Es JH, Giles RH and Clevers HC. (2001) The many faces of the tumor suppressor gene APC. *Experimental Cell Research.* 264(1): 126-134.

51. Kuhl M and Wedlich D. (1997) Wnt signalling goes nuclear. *Bioessays.* 19(2): 101-104.

52. Morin PJ. (1999) Beta-catenin signaling and cancer. *Bioessays.* 21(12): 1021-1030.

53. Orford K, Crockett C, Jensen JP *et al.* (1997) Serine phosphorylation-regulated ubiquitination and degradation of beta-catenin. *Journal of Biological Chemistry.* 272(40): 24735-24738.

54. Hedgepeth CM, Deardorff MA, Rankin K and Klein PS. (1999) Regulation of glycogen synthase kinase 3 beta and downstream Wnt signaling by axin. *Molecular and Cellular Biology.* 19(10): 7147-7157.

55. Yokoya F, Imamoto N, Tachibana T and Yoneda Y. (1999) Beta-catenin can be transported into the nucleus in a Ran-unassisted manner. *Molecular Biology of the Cell.* 10(4): 1119-1131.

56. Chung DC. (2000) The genetic basis of colorectal cancer: insights into critical pathways of tumorigenesis. *Gastroenterology.* 119: 854-65.

57. He TC, Sparks AB, Rago C *et al.* (1998) Identification of c-MYC as a target of the APC pathway. *Science.* 281(5382): 1509-1512.

58. Shtutman M, Zhurinsky J, Simcha I *et al.* (1999) The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proceedings of the National Academy of Sciences of the United States of America.* 96(10): 5522-5527.

59. Mann B, Gelos M, Siedow A *et al.* (1999) Target genes of beta-catenin-T cell-factor lymphoid-enhancer-factor signaling in human colorectal carcinomas. *Proceedings of the National Academy of Sciences of the United States of America.* 96(4): 1603-1608.

60. Zhang T, Otevrel T, Gao ZQ *et al.* (2001) Evidence that APC regulates survivin expression: A possible mechanism contributing to the stem cell origin of colon cancer. *Cancer Research.* 61(24): 8664-8667.

61. Brabletz T, Jung A, Dag S *et al.* (1999) Beta-catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. *American Journal of Pathology.* 155(4): 1033-1038.

62. Gradl D, Kuhl M and Wedlich D. (1999) The Wnt/Wg signal transducer beta-catenin controls fibronectin expression. *Molecular and Cellular Biology.* 19(8): 5576-5587.

63. He TC, Chan TA, Vogelstein B and Kinzler KW. (1999) PPAR delta is an APC-regulated target of nonsteroidal anti-inflammatory drugs. *Cell.* 99(3): 335-345.

64. Sparks AB, Morin PJ, Vogelstein B and Kinzler KW. (1998) Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. *Cancer Research.* 58(6): 1130-1134.

65. Vojtek AB and Der CJ. (1998) Increasing complexity of the Ras signaling pathway. *Journal of Biological Chemistry.* 273(32): 19925-19928.

66. Hedrick L, Cho KR, Fearon ER *et al.* (1994) The Dcc Gene-Product in Cellular-Differentiation and Colorectal Tumorigenesis. *Genes & Development.* 8(10): 1174-1183.

67. Massague J. (2000) How cells read TGF-beta signals. *Nature Reviews Molecular Cell Biology.* 1(3): 169-178.

68. Fridman JS and Lowe SW. (2003) Control of apoptosis by p53. *Oncogene.* 22(56): 9030-9040.

69. Taylor WR and Stark GR. (2001) Regulation of the G2/M transition by p53. *Oncogene.* 20: 1803-15.

70. Wang XW, Zhan Q, Coursen JD *et al.* (1999) Gadd induction of a G2/M cell cycle check point. *Proceedings of the National Academy of Sciences, USA.* 96: 3706-11.

71. Noda A, Ning Y, Venable SF *et al.* (1994) Cloning of Senescent Cell-Derived Inhibitors of DNA-Synthesis Using an Expression Screen. *Experimental Cell Research.* 211(1): 90-98.

72. Eldeiry WS, Tokino T, Velculescu VE *et al.* (1993) Waf1, a Potential Mediator of P53 Tumor Suppression. *Cell.* 75(4): 817-825.

73. Kastan MB, Zhan QM, Eldeiry WS *et al.* (1992) A Mammalian-Cell Cycle Checkpoint Pathway Utilizing P53 and Gadd45 Is Defective in Ataxia-Telangiectasia. *Cell.* 71(4): 587-597.

74. Kondo Y and Issa JPJ. (2004) Epigenetic changes in colorectal cancer. *Cancer and Metastasis Reviews.* 23(1): 29-39.

75. Goelz SE, Vogelstein B, Hamilton SR, and Feinberg AP. (1985) Hypomethylation of DNA from benign and malignant human colon neoplasms. *Science.* 228(4696): 187–190.

76. Waddell WR and Loughry RW. (1983) Sulindac for Polyposis of the Colon. *Journal of Surgical Oncology.* 24(1): 83-87.

77. Herendeen JM and Lindley C. (2003) Use of NSAIDs for the chemoprevention of colorectal cancer. *Annals of Pharmacotherapy.* 37(11): 1664-1674.

78. Brown WA, Skinner SA, Malcontenti-Wilson C *et al.* (2001) Non-steroidal anti-inflammatory drugs with activity against either cyclooxygenase 1 or cyclooxygenase 2 inhibit colorectal cancer in a DMH rodent model by inducing apoptosis and inhibiting cell proliferation. *Gut.* 48(5): 660-666.

79. Jacoby RF, Seibert K, Cole CE *et al.* (2000) The cyclooxygenase-2 inhibitor celecoxib is a potent preventive and therapeutic agent in the min mouse model of adenomatous polyposis. *Cancer Research.* 60(18): 5040-5044.

80. Yoshimi N, Shimizu M, Matsunaga K *et al.* (1999) Chemopreventive effect of N-(2-cyclohexyloxy-4-nitrophenyl)methane sulfonamide (NS-398), a selective cyclooxygenase-2 inhibitor, in rat colon carcinogenesis induced by azoxymethane. *Japanese Journal of Cancer Research.* 90(4): 406-412.

81. Ritland SR and Gendler SJ. (1999) Chemoprevention of intestinal adenomas in the Apc(Min) mouse by piroxicam: kinetics, strain effects and resistance to chemosuppression. *Carcinogenesis.* 20(1): 51-58.

82. Reddy BS, Rao CV and Seibert K. (1996) Evaluation of cyclooxygenase-2 inhibitor for potential chemopreventive properties in colon carcinogenesis. *Cancer Research.* 56(20): 4566-4569.

83. Reddy BS, Hirose Y, Lubet R *et al.* (2000) Chemoprevention of colon cancer by specific cyclooxygenase-2 inhibitor, celecoxib, administered during different stages of carcinogenesis. *Cancer Research.* 60(2): 293-297.

84. Boolbol SK, Dannenberg AJ, Chadburn A *et al.* (1996) Cyclooxygenase-2 overexpression and tumor formation are blocked by sulindac in a murine model of familial adenomatous polyposis. *Cancer Research.* 56(11): 2556-2560.

85. Huls G, Koornstra JJ and Kleibeuker JH. (2003) Non-steroidal anti-inflammatory drugs and molecular carcinogenesis of colorectal carcinomas. *Lancet.* 362(9379): 230-232.

86. Ashktorab H, Dawkins FW, Mohamed R *et al.* (2005) Apoptosis induced by aspirin and 5-fluorouracil in human colonic adenocarcinoma cells. *Digestive Diseases and Sciences.* 50(6): 1025-1032.

87. Pelzmann M, Thurnher D, Gedlicka C *et al.* (2004) Nimesulide and indomethacin induce apoptosis in head and neck cancer cells. *Journal of Oral Pathology & Medicine.* 33(10): 607-613.

88. Tarnawski AS and Jones MK. (2003) Inhibition of angiogenesis by NSAIDs: molecular mechanisms and clinical implications. *Journal of Molecular Medicine-Jmm.* 81(10): 627-636.

89. Cheong E, Ivory K, Doleman J *et al.* (2004) Synthetic and naturally occurring COX-2 inhibitors suppress proliferation in a human oesophageal adenocarcinoma cell line (OE33) by inducing apoptosis and cell cycle arrest. *Carcinogenesis.* 25(10): 1945-1952.

90. DuBois RN, Radhika A, Reddy BS and Entingh AJ. (1996) Increased cyclooxygenase-2 levels in carcinogen-induced rat colonic tumors. *Gastroenterology.* 110(4): 1259-1262.

91. Oshima M, Dinchuk JE, Kargman SL *et al.* (1996) Suppression of intestinal polyposis in Apc(Delta 716) knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell.* 87(5): 803-809.

92. Piazza GA, Alberts DS, Hixson LJ *et al.* (1997) Sulindac sulfone inhibits azoxymethane-induced colon carcinogenesis in rats without reducing prostaglandin levels. *Cancer Research.* 57(14): 2909-2915.

93. Hanif R, Pittas A, Feng Y *et al.* (1996) Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. *Biochemical Pharmacology.* 52(2): 237-245.

94. Hanif R, Pittas A, Feng Y, *et al.* (1995) Nsaids Inhibit the Growth of Colon-Cancer Cell-Lines by a Prostaglandin Independent Pathway. *Gastroenterology.* 108(4): A478-A478.

95. Druckrey H, Preussman R, Matzkies F and Ivankovic S. (1967) Erzeugung von Darmkrebs bei Ratten durch 1,2-dimethylhydrazin. *Naturewissenschaften.* 54: 285-286.

96. Druckrey H. (1968) Organotropic carcinogenesis and mechanisms of action of symmetrical dialkylhydrazines and azo-and azoalkanes. *Food Cosmet. Toxicol.* 6: 578-579.

97. Druckery H. (1970) Production of colonic carcinomas by 1,2-dialkylhydrazines and azoalkanes. In *Carcinoma of the colon and antecedent epithelium*, (WJ Burdette, ed.), Charles C Thomas, Springfield, IL, pp. 267-79.

98. Hong MY, Chapkin RS, Wild CP *et al.* (1999) Relationship between DNA adduct levels, repair enzyme, and apoptosis as a function of DNA methylation by azoxymethane. *Cell Growth & Differentiation.* 10(11): 749-758.

99. Hollstein M, Sidransky D, Vogelstein B and Harris CC. (1991) P53 Mutations in Human Cancers. *Science.* 253(5015): 49-53.

100. Takahashi M, Fukuda K, Sugimura T and Wakabayashi K. (1998) Beta-catenin is frequently mutated and demonstrates altered cellular location in azoxymethane-induced rat colon tumors. *Cancer Research.* 58(1): 42-46.

101. Jacoby RF, Llor X, Teng BB *et al.* (1991) Mutations in the K-Ras Oncogene Induced by 1,2-Dimethylhydrazine in Preneoplastic and Neoplastic Rat Colonic Mucosa. *Journal of Clinical Investigation.* 87(2): 624-630.

102. Vivona AA, Shpitz B, Medline A *et al.* (1993) K-Ras Mutations in Aberrant Crypt Foci, Adenomas and Adenocarcinomas During Azoxymethane-Induced Colon Carcinogenesis. *Carcinogenesis.* 14(9): 1777-1781.

103. Takahashi M and Wakabayashi K. (2004) Gene mutations and altered gene expression in azoxymethane-induced colon carcinogenesis in rodents. *Cancer Science.* 95(6): 475-480.

104. Watanabe K, Kawamori T, Nakatsugi S and Wakabayashi K. (2000) COX-2 and iNOS, good targets for chemoprevention of colon cancer. *Biofactors.* 12(1-4): 129-133.

105. Sandler RS. (1996) Epidemiology and risk factors for colorectal cancer. *Gastroenterol Clin North America.* 25(4): 717-35.

106. McKeown-Eyssen G. (1994) Epidemiology of colorectal cancer revisited: are serum triglycerides and/or plasma glucose associated with risk? *Cancer Epidemiology Biomarkers & Prevention.* 3(8): 687-95.

107. Giovannucci E. (1995) Insulin and Colon-Cancer. *Cancer Causes & Control.* 6(2): 164-179.

108. Bianchini F, Kaaks R and Vainio H. (2002) Overweight, obesity, and cancer risk. *Lancet Oncology.* 3(9): 565-574.

109. Chang CK and Ulrich CM. (2003) Hyperinsulinaemia and hyperglycaemia: possible risk factors of colorectal cancer among diabetic patients. *Diabetologia.* 46(5): 595-607.

110. Komninou D, Ayonote A, Richie JP and Rigas B. (2003) Insulin resistance and its contribution to colon carcinogenesis. *Experimental Biology and Medicine.* 228(4): 396-405.

111. Meyerkardt JA, Catalano PJ, Haller DG *et al.* (2003) Impact of diabetes mellitus on outcomes in patients with colon cancer. *Journal of Clinical Oncology.* 21(3): 433-440.

112. Kaaks R, Toniolo P, Akhmedkhanov A *et al.* (2000) Serum C-peptide, insulin-like growth factor (IGF)-I, IGF-binding proteins, and colorectal cancer risk in women. *Journal of the National Cancer Institute.* 92(19): 1592-1600.

113. Cross AJ and Sinha R. (2004) Meat-related mutagens/carcinogens in the etiology of colorectal cancer. *Environmental and Molecular Mutagenesis.* 44(1): 44-55.

114. Norat T, Lukanova A, Ferrari P and Riboli E. (2002) Meat consumption and colorectal cancer risk: Dose-response meta-analysis of epidemiological studies. *International Journal of Cancer.* 98(2): 241-256.

115. Malfatti MA, Shen NH, Wu RW *et al.* (1995) A Correlation of Salmonella Mutagenicity with DNA-Adducts Induced by the Cooked-Food Mutagen 2-Amino-1-Methyl-6-Phenylimidazo[4,5-B]Pyridine. *Mutagenesis.* 10(5): 425-431.

116. Ohgaki H, Takayama S and Sugimura T. (1991) Carcinogenicities of Heterocyclic Amines in Cooked Food. *Mutation Research.* 259(3-4): 399-410.

117. Hasegawa R, Sano M, Tamano S *et al.* (1993) Dose-Dependence of 2-Amino-1-Methyl-6-Phenylimidazo[4,5-B]-Pyridine (Phip) Carcinogenicity in Rats. *Carcinogenesis.* 14(12): 2553-2557.

118. Reddy BS. (1986) Diet and colon cancer: evidence from human and animal model studies. (Reddy B. S. Cohen L. A. eds.). In *Diet, Nutrition, and Cancer: A Critical Evaluation,* CRC Press Boca Raton, FL , pp. 47-65.

119. Hill MJ, Crowther JS, Drasar BS *et al.* (1971) Bacteria and the aetiology of cancer in the large bowel. *Lancet.* I: 95-100.

120. Velaquez OC, Zhou D, Seto RW *et al.* (1996) *In vivo* crypt surface hyperproliferation is decreased by butyrate and increased by deoxycholate in normal rat colon: associated *in vivo* effects on c-Fos and c-Jun expression. *J.P.E.N.* 20: 243-50.

121. Narisawa T, Magadia NE, Weinsburger JH and Wynder EL. (1974) Promoting effect of bile acids on colon carcinogenesis after intrarectal instillation of *N*-methyl-*N*-nitro-*N*-nitrosoguanidine in rats. *JNCI.* 53: 1093-97.

122. Reddy BS, Watanabe K, Weisburger JH and Wynder EL. (1977) Promoting effect of bile acids in colon carcinogenesis in germ free and conventional F344 rats. *Cancer Research.* 37: 3238-42.

123. Summerton J, Goeting N, Trotter GA and Taylor I. (1985) Effect of deoxycholic acid on the tumor incidence, distribution, and receptor status of colorectal cancer in the rat model. *Digestion.* 31: 77-81.

124. Beaty MM, Lee EY and Glauert HP. (1993) Influence of Dietary Calcium and Vitamin-D on Colon Epithelial-Cell Proliferation and 1,2-Dimethylhydrazine-Induced Colon Carcinogenesis in Rats Fed High-Fat Diets. *Journal of Nutrition.* 123(1): 144-152.

125. Lapre JA, Devries HT, Koeman JH and Vandermeer R. (1993) The Antiproliferative Effect of Dietary Calcium on Colonic Epithelium Is Mediated by Lumenal Surfactants and Dependent on the Type of Dietary-Fat. *Cancer Research.* 53(4): 784-789.

126. Lipkin M and Newmark H. (1985) Effect of added dietary calcium on colonic epithelial cell proliferation in subjects at high risk for familial colonic cancer. *New England Journal of Medicine.* 313: 1381-1384.

127. Rozen, PA, Fireman N, Fine *et al.* (1989) Oral calcium suppresses increased rectal epithelial proliferation of persons at risk for colorectal cancer. *Gut.* 30: 650-655.

128. Wargovich, MJ, Isbell G, Shabot M *et al.* (1992) Calcium supplementation decreases rectal epithelial cell proliferation in subjects with sporadic adenoma. *Gastroenterology.* 103: 92-97.

129. Holt PR, Wolper C, Moss SF *et al.* (2001) Comparison of calcium supplementation or low-fat dairy foods on epithelial cell proliferation and differentiation. *Nutrition and Cancer-an International Journal.* 41(1-2): 150-155.

130. Simanowski UA, Homann N, Knuhl M *et al.* (2001) Increased rectal cell proliferation following alcohol abuse. *Gut.* 49(3): 418-422.

131. Simanowski UA, Suter P, Russell RM *et al.* (1994) Enhancement of Ethanol-Induced Rectal Mucosal Hyper Regeneration with Age in F344 Rats. *Gut.* 35(8): 1102-1106.

132. Heavey PM, McKenna D and Rowland IR. (2004) Colorectal cancer and the relationship between genes and the environment. *Nutrition and Cancer-an International Journal.* 48(2): 124-141.

133. Giovannucci E, Rimm EB, Ascherio A *et al.* (1995) Alcohol, low methionine-low folate diets, and risk for colon cancer in men. *J Natl Cancer Int.* 87:265-273.

134. Su LJ and Arab L. (2001) Nutritional status of folate and colon cancer risk: Evidence from NHANES I Epidemiologic Follow-up Study. *Annals of Epidemiology.* 11(1): 65-72.

135. Groff JL and Gropper SS. (2000) Folic Acid. In *Advanced Nutrition and Human Metabolism, 3rd Edition*, West Publishing Company, St. Paul, MN, pp. 262-270.

136. Mason J and Choi SW. (2000) Folate and carcinogenesis: Developing a unifying hypothesis. *Advan Enzyme Regul.* 40:127-141.

137. Choi SW and Mason JB. (2002) Folate status: Effects on pathways of colorectal carcinogenesis. *Journal of Nutrition.* 132(8): 2413S-2418S.

138. Hoffman RM. (1984) Altered methionine metabolism, DNA methylation and oncongene expression in carcinogenesis: A review and synthesis. *Biochim Biophys Acta.* 738:49-87.

139. Kim YI, Shirwadkar S, Choi SW *et al.* (2000) Effects of dietary folate on DNA strand breaks within mutation-prone exons of the p53 gene in rat colon. *Gastroenterology.* 119(1): 151-161.

140. Slattery ML, Curtin K, Anderson K *et al.* (2000) Associations between dietary intake and Ki-ras mutations in colon tumors: A population-based study. *Cancer Research.* 60(24): 6935-6941.

141. Martinez ME, Maltzman T, Marshall JR *et al.* (1999) Risk factors for Ki-ras protooncogene mutation in sporadic colorectal adenomas. *Cancer Research.* 59(20): 5181-5185.

142. Steinmetz KA and Potter JD. (1991) Vegetables, fruit, and cancer. II. Mechanisms. *Cancer, Causes, and Control.* 2: 427-442.

143. Kennedy AR. (1993) Cancer Prevention by Protease Inhibitors. *Preventive Medicine.* 22(5): 796-811.

144. Kennedy AR. (1994) Prevention of Carcinogenesis by Protease Inhibitors. *Cancer Research.* 54(7): S1999-S2005.

145. Reddy BS and Rao CV. (1994) Chemoprevention of Colon-Cancer by Thiol and Other Organosulfur Compounds. *Food Phytochemicals for Cancer Prevention I.* 546: 164-172.

146. Smith T, Musk SRR and Johnson IT. (1996) Allyl isothiocyanate selectively kills undifferentiated HT29 cells in vitro and suppresses aberrant crypt foci in the colonic mucosa of rats. *Biochem. Soc. Trans.* 24: 381S.

147. Rijnkels JM, Hollanders VMH, Woutersen RA *et al.* (1997) Modulation of dietary fat-enhanced colorectal carcinogenesis in N-methyl-N'-nitro-N-nitrosoguanidine-treated rats by a vegetables-fruit mixture. *Nutrition and Cancer-an International Journal.* 29(1): 90-95.

148. Kennedy AR. (1998) The Bowman-Birk inhibitor from soybeans as an anticarcinogenic agent. *American Journal of Clinical Nutrition.* 68(6): 1406S-1412S.

149. Kennedy AR. (1998) Chemopreventive agents: Protease inhibitors. *Pharmacology & Therapeutics.* 78(3): 167-209.

150. Chung FL, Conaway CC, Rao CV and Reddy BS. (2000) Chemoprevention of colonic aberrant crypt foci in Fischer rats by sulforaphane and phenethylisothiocyanate. *Carcinogenesis.* 21(12): 2287-2291.

151. Seow A, Yuan JM, Sun CL *et al.* (2002) Dietary isothiocyanates, glutathione S-transferase polymorphisms and colorectal cancer risk in the Singapore Chinese Health Study. *Carcinogenesis.* 23(12): 2055-2061.

152. Emerit I. (1994) Reactive Oxygen Species, Chromosome Mutation, and Cancer - Possible Role of Clastogenic Factors in Carcinogenesis. *Free Radical Biology and Medicine.* 16(1): 99-109.

153. Gate L, Paul J, Ba GN *et al.* (1999) Oxidative stress induced in pathologies: the role of antioxidants. *Biomedicine & Pharmacotherapy.* 53(4): 169-180.

154. Zhu CY, Poulsen HE and Loft S. (2000) Inhibition of oxidative DNA damage in vitro by extracts of Brussels sprouts. *Free Radical Research.* 33(2): 187-196.

155. Feng Q, Kumagai T, Torii Y *et al.* (2001) Anticarcinogenic antioxidants as inhibitors against intracellular oxidative stress. *Free Radical Research.* 35(6): 779-788.

156. Bartsch H, Nair J and Owen RW. (2002) Exocyclic DNA adducts as oxidative stress markers in colon carcinogenesis: Potential role of lipid peroxidation, dietary fat and antioxidants. *Biological Chemistry.* 383(6): 915-921.

157. Flagg EW, Coates RJ and Greenberg RS. (1995) Epidemiologic Studies of Antioxidants and Cancer in Humans. *Journal of the American College of Nutrition.* 14(5): 419-427.

158. Neugut AI, Horvath K, Whelan RL *et al.* (1996) The effect of calcium and vitamin supplements on the incidence and recurrence of colorectal adenomatous polyps. *Cancer.* 78(4): 723-728.

159. Jacobs EJ, Connell CJ, Patel AV *et al.* (2001) Vitamin C and vitamin E supplement use and colorectal cancer mortality in a large American Cancer Society cohort. *Cancer Epidemiology Biomarkers & Prevention.* 10(1): 17-23.

160. Albanes D, Malila N, Taylor PR *et al.* (2000) Effects of supplemental alpha-tocopherol and beta-carotene on colorectal cancer: results from a controlled trial (Finland). *Cancer Causes & Control.* 11(3): 197-205.

161. Wu K, Willett WC, Chan JM *et al.* (2002) A prospective study on supplemental vitamin E intake and risk of colon cancer in women and men. *Cancer Epidemiology Biomarkers & Prevention.* 11(11): 1298-1304.

162. Malila N, Virtamo J, Virtanen M *et al.* (2002) Dietary and serum alpha-tocopherol, beta-carotene and retinol, and risk for colorectal cancer in male smokers. *European Journal of Clinical Nutrition.* 56(7): 615-621.

163. Chung H, Wu DY, Han SN *et al.* (2003) Vitamin E supplementation does not alter azoxymethane-induced colonic aberrant crypt foci formation in young or old mice. *Journal of Nutrition.* 133(2): 528-532.

164. Wang WQ and Higuchi CM. (1995) Induction of NAD(P)H:quinone reductase by vitamins A, E and C in colo205 colon cancer cells. *Cancer Letters.* 98(1): 63-69.

165. Guyonnet D, Siess MH, Le Bon AM and Suschetet M. (1999) Modulation of phase II enzymes by organosulfur compounds from allium vegetables in rat tissues. *Toxicology and Applied Pharmacology.* 154(1): 50-58.

166. Wilkinson J and Clapper ML. (1997) Detoxication enzymes and chemoprevention. *Proceedings of the Society for Experimental Biology and Medicine.* 216(2): 192-200.

167. Munday R and Munday CM. (2001) Relative activities of organosulfur compounds derived from onions and garlic in increasing tissue activities of quinone reductase and glutathione transferase in rat tissues. *Nutrition and Cancer-an International Journal.* 40(2): 205-210.

168. Begleiter A, Sivananthan K, Curphey TJ and Bird RP. (2003) Induction of NAD(P)H quinone : oxidoreductase1 inhibits carcinogen-induced aberrant crypt foci in colons of Sprague-Dawley rats. *Cancer Epidemiology Biomarkers & Prevention.* 12(6): 566-572.

169. St. Clair WH, Billings PC and Kennedy AR. (1990) The Effects of the Bowman-Birk Protease Inhibitor on C-Myc Expression and Cell-Proliferation in the Unirradiated and Irradiated Mouse Colon. *Cancer Letters.* 52(2): 145-152.

170. Chang JD, Li JH, Billings PC and Kennedy AR. (1990) Effects of Protease Inhibitors on C-Myc Expression in Normal and Transformed C3h-10t1/2 Cell-Lines. *Molecular Carcinogenesis.* 3(4): 226-232.

171. Billings PC and Habres JM. (1992) A Growth-Regulated Protease Activity That Is Inhibited by the Anticarcinogenic Bowman-Birk Protease Inhibitor. *Proceedings of the National Academy of Sciences of the United States of America.* 89(7): 3120-3124.

172. Mutoh H, Takahashi M, Fukuda K *et al.* (2000) Suppression by flavonoids of cyclooxygenase-2 promoter-dependent transcriptional activity in colon cancer cells: Structure-activity relationship. *Japanese Journal of Cancer Research.* 91(7): 686-691.

173. Wenzel H, Kuntz S, Brendel MD and Daniel H. (2000) Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells. *Cancer Research.* 60(14): 3823-3831.

174. Surh YJ, Chun KS, Cha HH *et al.* (2001) Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis.* 480(243-268.

175. Banerjee T, Van der Vliet A and Ziboh VA. (2002) Downregulation of COX-2 and iNOS by amentoflavone and quercetin in A549 human lung adenocarcinoma cell line. *Prostaglandins Leukotrienes and Essential Fatty Acids.* 66(5-6): 485-492.

176. Cheong E, Ivory K, Doleman J *et al.* (2004) Synthetic and naturally occurring COX-2 inhibitors suppress proliferation in a human oesophageal adenocarcinoma cell line (OE33) by inducing apoptosis and cell cycle arrest. *Carcinogenesis.* 25(10): 1945-1952.

177. Parnaud G, Li PF, Cassar G *et al.* (2004) Mechanism of sulforaphane-induced cell cycle arrest and apoptosis in human colon cancer cells. *Nutrition and Cancer-an International Journal.* 48(2): 198-206.

178. Jackson SJT and Singletary KW. (2004) Sulforaphane: a naturally occurring mammary carcinoma mitotic inhibitor, which disrupts tubulin polymerization. *Carcinogenesis.* 25(2): 219-227.

179. Bingham S. (1990) Mechanisms and experimental and epidemiological evidence relating dietary fibre (non-starch polysaccharides) and starch to protection against large bowel cancer. *Proc. Nutr. Soc.* 49(2): 152-157.

180. Reddy BS. (1992) Effect of dietary fiber on colonic bacterial enzymes and bile acids in relation to colon cancer. *Gastroenterology.* 102: 1475-82.

181. Cummings JH. (1981) Short chain fatty acids in the human colon. *Gut.* 22(9): 763-79.

182. Cummings JH and Macfarlane GT. (1991) The control and consequences of bacterial fermentation in the human colon. *J. Appl. Bacter.* 70: 443-59.

183. Heerdt BG, Houston MA and Augenlicht LH. (1997) Short-chain fatty acid-initiated cell cycle arrest and apoptosis of colonic epithelial cells is linked to mitochondrial function. *Cell Growth & Differentiation.* 8: 523-32.

184. Hague A, Manning AM, Huschtscha LI *et al.* (1993) Sodium butyrate induces apoptosis in human colonic tumor cell lines in a p-53 independent pathway: implications for the possible role of dietary fiber in the prevention of large bowel cancer. *Int. J. Cancer.* 55: 498-505.

185. Whitehead RH, Young GP and Bhatal PS. (1986) Effects of Short-Chain Fatty Acids on a new human colon carcinoma cell line (LIM1215). *Gut.* 27:1457-63.

186. Thorup I, Meyer O and Kristiansen E. (1995) Effects of potato starch, cornstarch, and sucrose on aberrant crypt foci in rats exposed to azoxymethane. *AnticancerResearch.* 15: 2101-2106.

187. Alabaster O, Tang ZC, Frost A and Shivapurkar N. (1995) Effect of Beta-Carotene and Wheat Bran Fiber on Colonic Aberrant Crypt and Tumor-Formation in Rats Exposed to Azoxymethane and High Dietary-Fat. *Carcinogenesis.* 16(1): 127-132.

188. Madar Z, Weiss O, Timar B *et al.* (1996) The effects of high-fiber diets on chemically induced colon cancer in rats. *Cancer Journal.* 9(4): 207-211.

189. Sakamoto J, Nakaji S, Sugawara K *et al.* (1996) Comparison of resistant starch with cellulose diet on 1,2-dimethylhydrazine-induced colonic carcinogenesis in rats. *Gastroenterology.* 110(1): 116-120.

190. Young GP, McIntyre A, Albert V *et al.* (1996) Wheat bran suppresses potato starch-potentiated colorectal tumorigenesis at the aberrant crypt stage in a rat model. *Gastroenterology.* 110(2): 508-514.

191. Zoran DL, Turner ND and Taddeo SS. (1997) Wheat bran diet reduces tumor incidence in a rat model of colon cancer independent of effects on distal luminal butyrate concentrations. *Journal of Nutrition.* 127: 2217-25.

192. Cassand, Pierrette, Maziere S *et al.* (1997) Effects of resistant starch and Vitamin A-supplemented diets on the promotion of precursor lesions of colon cancer in rats. *Nutrition and Cancer.* 27(1): 53-59.

193. Pierre F, Perrin P, Champ M *et al.* (1997) Short-chain fructo-oligosaccharides reduce the occurrence of colon tumors and gut-associated lymphoid tissue in *Min* mice. *Cancer Res.*57: 225-28.

194. Alabaster O, Tang Z and Shivapurkar N. (1997) Inhibition by wheat bran cereals of the development of aberrant crypt foci and colon tumours. *Food and Chemical Toxicology.* 35(5): 517-522.

195. Madar Z, Gurevich P, Ben-Hur H *et al.* (1998) Effects of dietary fiber on the rat intestinal mucosa exposed to low doses of a carcinogen. *Anticancer Research.* 18(5A): 3521-3526.

196. Rao CV, Chou D, Simi B *et al.* (1998) Prevention of colonic aberrant crypt foci and modulation of large bowel microbial activity by dietary coffee fiber, inulin and pectin. *Carcinogenesis.* 19(10): 1815-1819.

197. Verghese M, Rao DR, Chawan CB and Shackelford L. (2002) Dietary inulin suppresses azoxymethane-induced preneoplastic aberrant crypt foci in mature Fisher 344 rats. *Journal of Nutrition.* 132(9): 2804-2808.

198. Compher CW, Frankel WL, Tazelaar J *et al.* (1999) Wheat bran decreases aberrant crypt foci, preserves normal proliferation, and increases luminal butyrate levels in experimental colon cancer. *J.P.E.N.* 23: 269-78.

199. Lindner MA. (1991) A Fish Oil Diet Inhibits Colon Cancer in Mice. *Nutrition and Cancer-an International Journal.* 15(1): 1-11.

200. Chang WCL, Chapkin RS and Lupton JR. (1997) Predictive value of proliferation, differentiation and apoptosis as intermediate markers for colon tumorigenesis. *Carcinogenesis.* 18(4): 721-730.

201. Singh J, Hamid R and Reddy BS. (1998) Dietary fish oil inhibits the expression of farnesyl protein transferase and colon tumor development in rodents. *Carcinogenesis.* 19(6): 985-989.

202. Chang WCL, Chapkin RS and Lupton JR. (1998) Fish oil blocks azoxymethane-induced rat colon tumorigenesis by increasing cell differentiation and apoptosis rather than decreasing cell proliferation. *Journal of Nutrition.* 128(3): 491-497.

203. Coleman LJ, Landstrom EK, Royle PJ *et al.* (2002) A diet containing alpha-cellulose and fish oil reduces aberrant crypt foci formation and modulates other possible markers for colon cancer risk in azoxymethane-treated rats. *Journal of Nutrition.* 132(8): 2312-2318.

204. Covert KL, Newton AH, Turner ND *et al.* (2003) The combination of dietary fish oil and butyrate decreases high-multiplicity aberrant crypt foci in experimentally induced colon cancer. *Faseb Journal.* 17(5): A1154-A1154.

205. Singh J, Hamid R and Reddy BS. (1997) Dietary fat and colon cancer: Modulating effect of types and amount of dietary fat on ras-p21 function during promotion and progression stages of colon cancer. *Cancer Research.* 57(2): 253-258.

206. Davidson LA, Lupton JR, Jiang YH and Chapkin RS. (1999) Carcinogen and dietary lipid regulate ras expression and localization in rat colon without affecting farnesylation kinetics. *Carcinogenesis.* 20(5): 785-791.

207. Collett ED, Davidson LA, Fan YY *et al.* (2001) n-6 and n-3 polyunsaturated fatty acids differentially modulate oncogenic Ras activation in colonocytes. *American Journal of Physiology-Cell Physiology.* 280(5): C1066-C1075.

208. Murray NR, Weems C, Chen L *et al.* (2002) Protein kinase C beta 11 and TGF beta RII in omega-3 fatty acid-mediated inhibition of colon carcinogenesis. *Journal of Cell Biology.* 157(6): 915-920.

209. Bartram HP, Gostner A, Scheppach W *et al.* (1993) Effects of Fish-Oil on Rectal Cell-Proliferation, Mucosal Fatty-Acids, and Prostaglandin-E2 Release in Healthy-Subjects. *Gastroenterology.* 105(5): 1317-1322.

210. Rao CV, Simi B, Wynn TT *et al.* (1996) Modulating effect of amount and types of dietary fat on colonic mucosal phospholipase A(2), phosphatidylinositol-specific phospholipase C activities, and cyclooxygenase metabolite formation during different stages of colon tumor promotion in male F344 rats. *Cancer Research.* 56(3): 532-537.

211. Sandhu MS, Dunger DB and Giovannucci EL. (2002) Insulin, insulin-like growth factor-I (IGF-I), IGF binding proteins, their biologic interactions, and colorectal cancer. *Journal of the National Cancer Institute.* 94(13): 972-980.

212. Palmqvist R, Hallmans G, Rinaldi S *et al.* (2002) Plasma insulin-like growth factor 1, insulin-like growth factor binding protein 3, and risk of colorectal cancer: a prospective study in northern Sweden. *Gut.* 50(5): 642-646.

213. Renehan AG, O'Connell J, O'Halloran D *et al.* (2003) Acromegaly and colorectal cancer: A comprehensive review of epidemiology, biological mechanisms, and clinical implications. *Hormone and Metabolic Research.* 35(11-12): 712-725.

214. Macdonald RS, Thornton WH and Bean TL. (1993) Insulin and Igf-1 Receptors in a Human Intestinal Adenocarcinoma Cell-Line (Caco-2) - Regulation of Na+ Glucose-Transport across the Brush-Border. *Journal of Receptor Research.* 13(7): 1093-1113.

215. Guo YS, Narayan S, Yallampalli C and Singh P. (1992) Characterization of Insulin-Like Growth Factor-I Receptors in Human Colon Cancer. *Gastroenterology.* 102(4): 1101-1108.

216. Ouban A, Muraca P, Yeatman T and Coppola D. (2003) Expression and distribution of insulin-like growth factor-1 receptor in human carcinomas. *Human Pathology.* 34(8): 803-808.

217. Koenuma M, Yamori T and Tsuruo T. (1989) Insulin and Insulin-Like Growth Factor-I Stimulate Proliferation of Metastatic Variants of Colon Carcinoma-26. *Japanese Journal of Cancer Research.* 80(1): 51-58.

218. Bjork J, Nilsson J, Hultcrantz R and Johansson C. (1993) Growth-Regulatory Effects of Sensory Neuropeptides, Epidermal Growth-Factor, Insulin, and Somatostatin on the Nontransformed Intestinal Epithelial-Cell Line Iec-6 and the Colon-Cancer Cell-Line Ht-29. *Scandinavian Journal of Gastroenterology.* 28(10): 879-884.

219. Warren RS, Yuan H, Matli MR, Ferrara N and Donner DB. (1996) Induction of vascular endothelial growth factor by insulin-like growth factor 1 in colorectal carcinoma. *Journal of Biological Chemistry.* 271(46): 29483-29488.

220. Sekharam M, Zhao HY, Sun M *et al.* (2003) Insulin-like growth factor 1 receptor enhances invasion and induces resistance to apoptosis of colon cancer cells through the Akt/Bcl-x(L) pathway. *Cancer Research.* 63(22): 7708-7716.

221. Corpet DE, Peiffer G and Tache S. (1998) Glycemic index, nutrient density, and promotion of aberrant crypt foci in rat colon. *Nutrition and Cancer-an International Journal.* 32(1): 29-36.

222. Tran TT, Medline A and Bruce WR. (1996) Insulin promotion of colon tumors in rats. *Cancer Epidemiology Biomarkers & Prevention.* 5(12): 1013-1015.

223. Koohestani N, Chia MC, Pham NA *et al.* (1998) Aberrant crypt focus promotion and glucose intolerance: correlation in the rat across diets differing in fat, n-3 fatty acids and energy. *Carcinogenesis.* 19(9): 1679-1684.

224. Geil PB and Anderson JW. (1994) Nutrition and Health Implications of Dry Beans - a Review. *Journal of the American College of Nutrition.* 13(6): 549-558.

225. Messina MJ. (1999) Legumes and soybeans: overview of their nutritional profiles and health effects. *American Journal of Clinical Nutrition.* 70(3): 439S-450S.

226. Anderson JW, Smith BM and Washnock CS. (1999) Cardiovascular and renal benefits of dry bean and soybean intake. *American Journal of Clinical Nutrition.* 70(3): 464S-474S.

227. Setchell KDR and Radd S. (2000) Soy and other legumes: 'Bean' around a long time but are they the 'superfoods' of the millennium and what are the safety issues for their constituent phytoestrogens? *Asia Pacific Journal of Clinical Nutrition.* 9:S13-S22.

228. Messina M and Messina V. (1991) Increasing Use of Soyfoods and Their Potential Role in Cancer Prevention. *Journal of the American Dietetic Association.* 91(7): 836-840.

229. Endres JG. (2001) Soy protein products: Characteristics, Nutritional Aspects, and Utilization, (Endres JG, ed.), AOCS Press, Champaign, IL, pp. 4-40.

230. US Department of Agriculture, Agricultural Research Service. (1999) USDA Nutrient database for standard reference, release 13. Nutrient data laboratory home page, www.nal.usda.gov/fnic/foodcomp.

231. Kushi LH, Meyer KA and Jacobs DR. (1999) Cereals, legumes, and chronic disease risk reduction: evidence from epidemiologic studies. *American Journal of Clinical Nutrition.* 70(3): 451S-458S.

232. Ludwig DDS. (2002) The glycemic index - Physiological mechanisms relating to obesity, diabetes, and cardiovascular disease. *Jama-Journal of the American Medical Association.* 287(18): 2414-2423.

233. Simpson HCR, Lousley S, Geekie M *et al.* (1981) A High-Carbohydrate Leguminous Fiber Diet Improves All Aspects of Diabetic Control. *Lancet.* 1(8210): 1-4.

234. Jenkins DJA, Wolever TMS, Jenkins AL *et al.* (1983) The Glycemic Index of Foods Tested in Diabetic-Patients - a New Basis for Carbohydrate Exchange Favoring the Use of Legumes. *Diabetologia.* 24 (4): 257-264.

235. Dilawari JB, Kumar VKA, Khurana S *et al.* (1987) Effect of Legumes on Blood-Sugar in Diabetes-Mellitus. *Indian Journal of Medical Research.* 85:184-187.

236. Viswanathan M, Ramachandran A, Indira P *et al.* (1989) Responses to Legumes in Niddm Subjects - Lower Plasma-Glucose and Higher Insulin Levels. *Nutrition Reports International.* 40(4): 803-812.

237. Jenkins DJA, Wong GS, Patten R *et al.* (1983) Leguminous Seeds in the Dietary-Management of Hyperlipidemia. *American Journal of Clinical Nutrition.* 38(4): 567-573.

238. Hu FB, Rimm EB, Stampfer MJ *et al.* (2000) Prospective study of major dietary patterns and risk of coronary heart disease in men. *American Journal of Clinical Nutrition.* 72: 912-921.

239. Bazzano LA, He J, Ogden LG *et al.* (2001) Legume consumption and risk of coronary heart disease in US men and women. *Archives of Internal Medicine.* 161: 2573-2578.

240. Fung TT, Willett WC, Stampfer MJ *et al.* (2001) Dietary patterns and the risk of coronary heart disease in women. *Archives of Internal Medicine.* 161: 1857-1862.

241. Bazzano LA, He J, Ogden LG *et al.* (2001) Legume consumption and risk of coronary heart disease in US men and women. *Archives of Internal Medicine.* 161(21): 2573-2578.

242. Leathwood P and Pollet P. (1988) Effects of slow release carbohydrates in the form of bean flakes on the evolution of hunger and satiety in man. *Appetite.* 10: 1-11.

243. Ludwig DS, Majzoub JA, Al Zahrani A *et al.* (1999) High glycemic index foods, overeating, and obesity. *Pediatrics.* 103(3):

244. Ludwig DS, Spieth LE, Lenders CM *et al.* (2000) A low glycemic index diet in the treatment of obesity. *Pediatric Research.* 47(4): 134A-134A.

245. Steinmetz KA and Potter JD. (1993) Food-group consumption and colon cancer in the Adelaide Case-Control Study. I. Vegetables and fruit. *Int J Cancer.* 53(5): 711-9.

246. Benito E, Stiggelbout A, Bosch FX *et al.* (1991) Nutritional factors in colorectal cancer risk: a case-control study in Majorca. *Int J Cancer.* 49(2): 161-7.

247. Messina M and Bennink MR. (1998) Soyfoods, isoflavones and risk of colonic cancer: a review of the in vitro and in vivo data. *Baillieres Clin Endocrinol Metab.* (4): 707-28.

248. Spector D, Anthony M, Alexander D and Arab L. (2003) Soy consumption and colorectal cancer. *Nutr Cancer.* 47(1): 1-12.

249. Nishi M, Yoshida K, Hirata K, and Miyake H. (1997) Eating habits and colorectal cancer. *Oncol Reports.* 4: 995–998.

250. Nagata C. (2000) Ecological study of the association between soy product intake and mortality from cancer and heart disease in Japan. *Int Epidemiol Assoc.* 29: 832–836.

251. McKeown-Eyssen GE and Bright-See E. (1984) Dietary factors in colon cancer: international relationships. *Nutr Cancer.* 6: 160–170.

252. Haenszel W, Berg JW, Segi M *et al.* (1973) Large bowel cancer in Hawaiian Japanese. *JNCI.* 51: 1765–1779.

253. Tajima K and Tominaga S. (1985) Dietary habits and gastro-intestinal cancers: a comparative case-control study of stomach and large intestinal cancers in Nagoya, Japan. *Jpn J Cancer Res.* 76: 705–716.

254. Hirayama T. (1990) Contribution of a long-term prospective cohort study to the issue of Nutrition and cancer with special reference to the role of alcohol drinking. *Prog Clin Biol Res.* 346: 179–187.

255. Hu J, Liu Y, Yu Y *et al.* (1991) Diet and cancer of the colon and rectum: a case-control study in China. *Int J Epidemiol.* 20: 362–367.

256. Hoshiyama Y, Sekine T and Sasaba T. (1993) A case-control study of colorectal cancer and its relation to diet, cigarettes, and alcohol consumption in Saitama Prefecture, Japan. *Tohoku J Exp Med.* 171: 153–165.

257. Kono S, Imanishi K, Shinchi K and Yanai F. (1993) Relationship of diet to small and large adenomas of the sigmoid colon. *Jpn J Cancer Res.* 84: 13–19.

258. Inoue M, Tajima K, Hirose K *et al.* (1995) Subsite-specific risk factors for colorectal cancer: a hospital-based case-control study in Japan. *Cancer Cause Control.* 6: 14–22.

259. Witte JS, Longnecker MP, Bird CL *et al.* (1996) Relation of vegetable, fruit, and grain consumption to colorectal adenomatous polyps. *Am J Epidemiol.* 144: 1015–1025.

260. Le Marchand L, Hankin JH, Wilkens LR *et al.* (1997) Dietary fiber and colorectal cancer risk. *Epidemiology.* 8: 658–665.

261. Nishi M, Yoshida K, Hirata K, and Miyake H. (1997) Eating habits and colorectal cancer. *Oncol Rep.* 4: 995–998.

262. Bennink MR. (2001) Dietary soy reduces colon carcinogenesis in human and rats - Soy and colon cancer. *Nutrition and Cancer Prevention.* 492: 11-17.

263. Thiagarajan DG, Bennink MR, Bourquin LD and Kavas FA. (1998) Prevention of precancerous colonic lesions in rats by soy flakes, soy flour, genistein, and calcium. *American Journal of Clinical Nutrition.* 68(6): 1394S-1399S.

264. McIntosh GH, Regester GO, Leleu RK *et al.* (1995) Dairy Proteins Protect against Dimethylhydrazine-Induced Intestinal Cancers in Rats. *Journal of Nutrition.* 125(4): 809-816.

265. Rondini EA and Bennink MR. (2002) Defatted soy flour, but not soy concentrate, reduces azoxymethane-induced colon carcinogenesis. *Journal of Nutrition.* 132(3): 589S-589S.

266. Helms JR and Gallagher DD. (1995) The effect of dietary soy protein isolate and genistein on the development of preneoplastic lesions (aberrant crypts) in rats. *Journal of Nutrition.* 125: 802S.

267. Gallagher DD, Gallagher CM, An ZL and Hoffman RM. (1998) Soy protein isolates and genistein: effects on initiation, promotion, and progression of colon cancer. *American Journal of Clinical Nutrition.* 68(6): 1524S-1524S.

268. Gee JM, Noteborn H, Polley ACJ and Johnson IT. (2000) Increased induction of aberrant crypt foci by 1,2- dimethylhydrazine in rats fed diets containing purified genistein or genistein-rich soya protein. *Carcinogenesis.* 21(12): 2255-2259.

269. Hakkak R, Korourian S, Ronis MJJ *et al.* (2001) Soy protein isolate consumption protects against azoxymethane- induced colon tumors in male rats. *Cancer Letters.* 166(1): 27-32.

270. Weed HG, McGandy RB and Kennedy AR. (1985) Protection against Dimethylhydrazine-Induced Adenomatous Tumors of the Mouse Colon by the Dietary Addition of an Extract of Soybeans Containing the Bowman-Birk Protease Inhibitor. *Carcinogenesis.* 6(8): 1239-1241.

271. Billings PC, Newberne PM and Kennedy AR. (1990) Protease Inhibitor Suppression of Colon and Anal Gland Carcinogenesis Induced by Dimethylhydrazine. *Carcinogenesis.* 11(7): 1083-1086.

272. Kennedy AR, Billings PC, Wan XS and Newberne PM. (2002) Effects of Bowman-Birk inhibitor on rat colon carcinogenesis. *Nutrition and Cancer-an International Journal.* 43(2): 174-186.

273. Shamsuddin AM, Elsayed AM and Ullah A. (1988) Suppression of Large Intestinal Cancer in F344 Rats by Inositol Hexaphosphate. *Carcinogenesis.* 9(4): 577-580.

274. Shamsuddin AM and Ullah A. (1989) Inositol Hexaphosphate Inhibits Large Intestinal Cancer in F344 Rats 5 Months after Induction by Azoxymethane. *Carcinogenesis.* 10(3): 625-626.

275. Ullah A and Shamsuddin AM. (1990) Dose-Dependent Inhibition of Large Intestinal Cancer by Inositol Hexaphosphate in F344 Rats. *Carcinogenesis.* 11(12): 2219-2222.

276. Graf E and Eaton JW. (1993) Suppression of Colonic-Cancer by Dietary Phytic Acid. *Nutrition and Cancer-an International Journal.* 19(1): 11-19.

277. Rao CV, Wang CX, Simi B *et al.* (1997) Enhancement of experimental colon cancer by genistein. *Cancer Research.* 57(17): 3717-3722.

278. Sorensen IK, Kristiansen E, Mortensen A *et al.* (1998) The effect of soy isoflavones on the development of intestinal neoplasia in Apc(Min) mouse. *Cancer Letters.* 130(1-2): 217-225.

279. Guo JY, Li XS, Browning JD *et al.* (2004) Dietary soy isoflavones and estrone protect ovariectomized ER alpha KO and wild-type mice from carcinogen-induced colon cancer. *Journal of Nutrition.* 134(1): 179-182.

280. Koratkar R and Rao AV. (1997) Effect of soya bean saponins on azoxymethane-induced preneoplastic lesions in the colon of mice. *Nutrition and Cancer-an International Journal.* 27(2): 206-209.

281. Raicht RF, Cohen BI, Fazzini EP *et al.* (1980) Protective effect of plant sterols against chemically induced colon tumors in rats. *Cancer Research.* 40(2): 403-5.

282. Witschi H and Kennedy AR. (1989) Modulation of Lung-Tumor Development in Mice with the Soybean-Derived Bowman-Birk Protease Inhibitor. *Carcinogenesis.* 10(12): 2275-2277.

283. Vucenik I, Sakamoto K, Bansal M and Shamsuddin AM. (1993) Inhibition of Rat Mammary Carcinogenesis by Inositol Hexaphosphate (Phytic Acid) - a Pilot-Study. *Cancer Letters.* 75(2): 95-102.

284. Vucenik I, Yang GY and Shamsuddin AM. (1995) Inositol Hexaphosphate and Inositol Inhibit Dmba-Induced Rat Mammary-Cancer. *Carcinogenesis.* 16(5): 1055-1058.

285. Vucenik I, Yang G and Shamsuddin AM. (1997) Comparison of pure inositol hexaphosphate and high-bran diet in the prevention of DMBA-induced rat mammary carcinogenesis. *Nutrition and Cancer-an International Journal.* 28(1): 7-13.

286. Estensen RD and Wattenberg LW. (1993) Studies of Chemopreventive Effects of Myoinositol on Benzo[a]Pyrene-Induced Neoplasia of the Lung and Forestomach of Female a/J Mice. *Carcinogenesis.* 14(9): 1975-1977.

287. Gupta KP, Singh J and Bharathi R. (2003) Suppression of DMBA-induced mouse skin tumor development by inositol hexaphosphate and its mode of action. *Nutrition and Cancer-an International Journal.* 46(1): 66-72.

288. Vucenik I, Zhang ZS and Shamsuddin AM. (1998) IP6 in treatment of liver cancer II. Intra-tumoral injection of IP6 regresses pre-existing human liver cancer xenotransplanted in nude mice. *Anticancer Research.* 18(6A): 4091-4096.

289. Vucenik I, Tomazic VJ, Fabian D and Shamsuddin AM. (1992) Antitumor-Activity of Phytic Acid (Inositol Hexaphosphate) in Murine Transplanted and Metastatic Fibrosarcoma, a Pilot-Study. *Cancer Letters.* 65(1): 9-13.

290. Billings PC, Stclair WH, Maki PA and Kennedy AR. (1992) Distribution of the Bowman Birk Protease Inhibitor in Mice Following Oral-Administration. *Cancer Letters.* 62(3): 191-197.

291. Billings PC, Brandon DL and Habres JM. (1991) Internalization of the Bowman-Birk Protease Inhibitor by Intestinal Epithelial-Cells. *European Journal of Cancer.* 27(7): 903-908.

292. Messadi DV, Billings P, Shklar G and Kennedy AR. (1986) Inhibition of Oral Carcinogenesis by a Protease Inhibitor. *Journal of the National Cancer Institute.* 76(3): 447-452.

293. Stclair WH, Billings PC and Kennedy AR. (1990) The Effects of the Bowman-Birk Protease Inhibitor on C-Myc Expression and Cell-Proliferation in the Unirradiated and Irradiated Mouse Colon. *Cancer Letters.* 52(2): 145-152.

294. Vucenik I and Shamsuddin AM. (2003) Cancer inhibition by inositol hexaphosphate (IP6) and inositol: From laboratory to clinic. *Journal of Nutrition.* 133(11): 3778S-3784S.

295. Sandra F, Matsuda M, Yoshida H and Hirata M. (2002) Inositol hexakisphosphate blocks tumor cell growth by activating apoptotic machinery as well as by inhibiting the Akt/NF kappa B-mediated cell survival pathway. *Carcinogenesis.* 23(12): 2031-2041.

296. El-Sherbiny YM, Cox MC, Ismail ZA *et al.* (2001) G(0)/G(1) arrest and S phase inhibition of human cancer cell lines by inositol hexaphosphate (IP6). *Anticancer Research.* 21(4A): 2393-2403.

297. Singh RP, Agarwal C and Agarwal R. (2003) Inositol hexaphosphate inhibits growth, and induces G1 arrest and apoptotic death of prostate carcinoma DU145 cells: modulation of CDKI-CDK-cyclin and pRb-related protein-E2F complexes. *Carcinogenesis.* 24(3): 555-563.

298. Jariwalla RJ, Sabin R, Lawson S *et al.* (1999) Dietary intrinsic phytate protects colon from lipid peroxidation in pigs with a moderately high dietary iron intake. *Proceedings of the Society for Experimental Biology and Medicine.* 221(1): 80-86.

299. Nelson RL. (1992) Dietary Iron and Colorectal-Cancer Risk. *Free Radical Biology and Medicine.* 12(2): 161-168.

300. Pagliacci MC, Smacchia M, Migliorati G *et al.* (1994) Growth-Inhibitory Effects of the Natural Phyto-Estrogen Genistein in Mcf-7 Human Breast-Cancer Cells. *European Journal of Cancer.* 30A(11): 1675-1682.

301. Peterson G and Barnes S. (1996) Genistein inhibits both estrogen and growth factor-stimulated proliferation of human breast cancer cells. *Cell Growth & Differentiation.* 7(10): 1345-1351.

302. Choi YH, Zhang LJ, Lee WH and Park KY. (1998) Genistein-induced G2/M arrest is associated with the inhibition of cyclin B1 and the induction of p21 in human breast carcinoma cells. *International Journal of Oncology.* 13(2): 391-396.

303. Zhou JR, Mukherjee P, Gugger ET *et al.* (1998) Inhibition of murine bladder tumorigenesis by soy isoflavones via alterations in the cell cycle, apoptosis, and angiogenesis. *Cancer Research.* 58(22): 5231-5238.

304. Li YW, Upadhyay S, Bhuiyan M and Sarkar FH. (1999) Induction of apoptosis in breast cancer cells MDA-MB-231 by genistein. *Oncogene.* 18(20): 3166-3172.

305. Salti GI, Grewal S, Mehta RR *et al.* (2000) Genistein induces apoptosis and topoisomerase II-mediated DNA breakage in colon cancer cells. *European Journal of Cancer.* 36(6): 796-802.

306. Nguyen DT, Garcia J and Cadenas E. (2001) Genistein causes cell cycle arrest and apoptosis via inhibition of p53-independent mechanisms in T47D breast cancer cells. *Free Radical Biology and Medicine.* 31(S105-S105.

307. Yu ZL, Li WJ and Liu FY. (2004) Inhibition of proliferation and induction of apoptosis by genistein in colon cancer HT-29 cells. *Cancer Letters.* 215(2): 159-166.

308. Yu ZL, Tang YN, Hu DS and Ll J. (2005) Inhibitory effect of genistein on mouse colon cancer MC-26 cells involved TGF-beta 1/Smad pathway. *Biochemical and Biophysical Research Communications.* 333(3): 827-832.

309. Kerry N and Abbey M. (1998) The isoflavone genistein inhibits copper and peroxyl radical mediated low density lipoprotein oxidation *in vitro. Atherosclerosis.* 140(2): 341-347.

310. Boadi WY, Iyere PA and Adunyah SE. (2005) *In vitro* exposure to quercetin and genistein alters lipid peroxides and prevents the loss of glutathione in human progenitor mononuclear (U937) cells. *Journal of Applied Toxicology.* 25(1): 82-88.

311. Fotsis T, Pepper M, Adlercreutz H *et al.* (1993) Genistein, a dietary-derived inhibitor of in vitro angiogenesis. *Proc Natl Acad Sci U S A.* 90: 2690-2694

312. Fotsis T, Pepper MS, Aktas E *et al.* (1997) Flavonoids, dietary-derived inhibitors of cell proliferation and *in vitro* angiogenesis. *Cancer Research.* 57: 2916-2921.

313. Wietrzyk J, Boratynski J, Grynkiewicz G *et al.* (2001) Antiangiogenic and antitumour effects *in vivo* of genistein applied alone or combined with cyclophosphamide. *Anticancer Research.* 21(6A): 3893-3896.

314. Buchler P, Reber HA, Buchler MW *et al.* (2004) Antiangiogenic activity of genistein in pancreatic carcinoma cells is mediated by the inhibition of hypoxia-inducible factor-1 and the down-regulation of VEGF gene expression. *Cancer.* 100(1): 201-210.

315. Akiyama T, Ishida J, Nakagawa S *et al.* (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J Biol Chem.* 262: 5592-5595.

316. Davidson LA, Nguyen DV, Hokanson RM *et al.* (2004) Chemopreventive n-3 polyunsaturated fatty acids reprogram genetic signatures during colon cancer initiation and progression in the rat. *Cancer Res.* 64(18): 6797-804.

317. Xiao R, Badger TM, Simmen FA. (2004) Dietary exposure to soy or whey proteins alters colonic global gene expression profiles during rat colon tumorigenesis. *Mol Cancer.* 4(1): 1-17.

CHAPTER III.

PROFILE OF GLOBAL GENE PATTERNS ALTERED IN AZOXYMETHANE (AOM)-INDUCED COLON TUMORS COMPARED TO NORMAL-APPEARING MUCOSA

A. ABSTRACT.

The azoxymethane (AOM)-treated rodent model has been utilized extensively for studying the etiology and molecular pathogenesis of colon cancer. This study was conducted to gain further insight into cellular and environmental differences in gene changes underlying AOM-induced colon cancer progression using microarrays. Male F344 rats were obtained at 3 weeks of age and fed a modified AIN-93G diet. At 4 and 5 weeks of age, animals were administered subcutaneous injections of AOM prepared in saline (15 mg/kg) to induce colon tumors. Animals were sacrificed at 36 weeks of age and microarrays were performed on mRNA isolated from tumorous tissue and surrounding non-neoplastic mucosa. Of the 8799 genes or ESTs present on RGU34A rat genome chips, we identified 471 transcripts (306 over-expressed and 165 under-expressed) significantly altered in tumors compared to normal colon tissue. A majority of genes (15%) induced during cancer progression were related to inflammatory and immune responses with evidence of tissue damage, remodeling, angiogenesis as well as a lower production of cytokines involved in anti-tumor immunity. A lower expression of ion transporters and metabolic enzymes highlights cellular disturbances in ion and water absorption and oxidative mitochondrial metabolism during cancer progression.

B. INTRODUCTION.

The development of CRC is a multi-stage process characterized by the accumulation of critical genetic and epigenetic events (1-2). For a majority of CRC, these changes culminate in the transformation of normal epithelium to adenomatous polyps and eventually malignant carcinomas (1-2). Critical mutations or gene deletions involved in the adenoma-carcinoma sequence in humans have been identified (1-2) and downstream signaling events are beginning to be explored in more detail (3-7). Additionally the association of chronic inflammation to risk of colon cancer is well documented (8-9), and the importance of communication and signaling between colonocytes and the extracellular matrix in maintaining normal mucosal function is becoming more recognized (4-7). However, despite being one of the earliest cancers by which a genetic model was proposed, CRC remains the second leading cause of cancer mortality in the US (10), indicating incomplete understanding of events involved in neoplastic transformation and metastases.

A majority of sporadic CRC cases are thought to be attributable to environmental exposures. Because the process of CRC can take up to 20 years in humans to fully manifest, animals are frequently utilized to screen for and examine molecular mechanisms whereby chemopreventative agents, including diet, can prevent or delay the cancer process (12). Druckery and collegues (13-15) were first to demonstrate that administration of the alkylating agent 1,2-dimethylhydrazine (DMH) or its active metabolite, azoxymethane (AOM) specifically and reliably induces colon cancer in rodents, and colon cancers induced in this model have been demonstrated to share similar molecular and pathological features to those in humans (11, 16). Although *APC*

mutations are rare (17), mutational activation and nuclear translocation of β-catenin have been reported (18). Additionally, mutations on codon 12 and 13 of the k-*ras* gene (19) accompanied by up-regulation of cyclo-oxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) are other features common to human cancers and in the AOM model (20-22).

This study was conducted to more thoroughly identify environmental and molecular changes underlying AOM-induced colon cancer as well as potential biomarkers involved in cancer progression. Male F344 rats, a highly susceptible strain, were subject to a long-term carcinogenesis study, and at 31 weeks following AOM administration, a time point when adenocarcinomas had developed, microarrays were performed on tumor tissue and surrounding non-neoplastic mucosa. The profile of genes differentially expressed and the biological relevance of some of the changes to human colon cancer are discussed.

C. MATERIALS AND METHODS.

Experimental protocol and tissue collection.

The tissues utilized in this experiment were obtained from part of a larger colon cancer study. Briefly, 3-week old male F344 rats (Harlan Sprague-Dawley, Indianapolis, IL) were fed a modified AIN-93G diet containing 11% dietary fiber, 16.7% fat, and 18.9% of protein as high nitrogen casein. After a one-week acclimatization period, rats received subcutaneous injections of azoxymethane (AOM, 15 mg/kg) prepared in saline once per week for two weeks. At 36 weeks of age rats were sacrificed and colons were resected, opened longitudinally, and rinsed briefly in tap water to remove debris. Polypoid lesions, when present, were excised from the distal half of the colon with a

razor blade. Epithelial cells were then collected by gently scraping the remaining normal-appearing mucosa from the distal half of the colon with the edge of a glass slide. All samples were snap frozen on dry ice immediately after collection and stored at –80 °C until needed.

Throughout the experiment, animals had free access to diet and distilled water and were assessed daily for health status and monthly for weight gain. The procedures used in this study were in accordance with the regulatory guidelines of the Michigan State University Committee on Animal Use and Care.

Microarray target preparation and hybridization.

For total RNA isolation, < 100 mg colonic mucosa or tumorous tissue was homogenized using a Tekmar homogenizer in one mL of TRIzol reagent containing 2 μL of RNase-free glycogen according to the manufacturers instructions (Gibco, Carlsbad, CA). Following extraction, RNA was purified with RNeasy mini columns (Qiagen, Valencia, CA), quantified using a UV spectrophotometer (A260/A280), and the quality was assessed by agarose-formaldehyde gel electrophoresis. Prior to cDNA synthesis, RNA was pooled from 2-4 animals/tissue type (tumors, normal mucosa) to control for biological variation in gene expression.

Biotinylated cRNA was prepared from high quality RNA (10 μg) in accordance with instructions supplied in the GeneChip Expression Manual (Affymetrix, Santa Clara, CA). Approximately 15 μg cRNA was then fragmented at 94 °C for 35 minutes and hybridized to RGU34A rat genome chips for 16 hours at 45 °C. Following hybridization, arrays were washed and stained with a streptavidin-phycoerythrin conjugate on an

Affymetrix Fluidics station according to standard protocol. Processed arrays were scanned at 570 nm using a Hewlett Packard GeneArray Scanner.

Quantitative reverse transcription-PCR (RT-PCR) analysis

The pooled RNA samples used for oligonucleotide microarrays were also used to confirm gene changes with quantitative real time polymerase chain reaction (RT-PCR). Gene specific primers for (β-Actin, group IIa secretory phospholipase A2 (sPLA2), NP defensin 3α, lysozyme, and deleted in malignant brain tumors (DMBT1) were designed with the Primer Express 2.0 program (**TABLE 1**; Applied Biosystems). Single-stranded (ss) cDNA was synthesized from 2.5 μg of total RNA using T7-(dT)24 primers (Proligo, Boulder, CO) and the Superscript II system (Invitrogen, Carlsbad, CA). Reverse transcription was performed in a thermocycler following the Superscript first strand synthesis protocol with the following modifications: temperature of reaction was cooled to 20 °C for 5 minutes following annealing of primer and ss cDNA synthesis time was increased to 60 minutes for 42 °C.

Quantitative determination of gene expression was performed with the ABIPrism7000 (Perkin Elmer Corp., Foster City, CA) using the Syber Green Universal Master Mix (Applied Biosystems). The reaction mixture (25 μL total volume) contained 20 ng ss cDNA, 12.5 μL SYBR Green RT-PCR Master mix, and 10.5 μL of diluted primers (forward and reverse). Primer pairs were used at a final concentration of 1.2 μM. The real-time cycle conditions were as follows: PCR initial activation step at 95 °C for 15 min and a total of 40 cycles for melting (95 °C, 15 s) and annealing/extension (60 °C, 1 min). All assays were performed in duplicates, using 2-3 samples per group.

Statistical analysis methods.

Data for microarrays and RT-PCR were analyzed using the General Linear Model procedure of SAS (SAS Institute, Inc. Cary, NC, Version 7.0), and when appropriate, individual comparisons were made using the least significant difference (LSD) method. Prior to statistical analyses, microarrays were globally scaled to a target intensity of 500 in Affymetrix Microarray Suite, version 5.0 to control within-chip variations and then imported into GeneSpring (Silicon Genetics, Inc., Redwood City, CA, Version 6.0) for normalization and filtering. All chips were normalized to the median intensity of invariant genes, defined as genes displaying less than a 30% coefficient of variation (CV) across tissue types (mucosa, tumors). A series of filtering steps were then performed to limit analysis of genes to those reliably measured and likely of physiological significance. The first filtering step excluded genes not considered "Present" or "Marginal" in at least 33% (2 out of 6) of the samples, reducing the gene list from 8799 to 4404 genes (50% of genes present on the chip). An additional filtering step limited the genes further to those exhibiting greater than 2 fold- or less than 0.5 mean fold-change difference between tissue types (875 genes). Normalized expression values were then analyzed using the GLM procedure of SAS.

Differentially expressed transcripts ($P < 0.05$) were then broadly categorized according to biological processes using gene ontologies retrieved from the Affymetrix NetAffix Analysis Center (http://www.affymetrix.com/analysis/index.affx) and the Rat Genome Database (RGD) gene annotation tool (Rat Genome Database Web Site, Medical

TABLE 1. Primer pairs used for RT-PCR. [1]

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
β-Actin	AACCGTGAAAAGATGACCCAGAT	CACAGCCTGGATGGCTACGT
Lyzozyme	AACTCTGAAAAGGAATGGGATGTC	TGCTCTCATGCTGAGCTAAACAC
NP defensin 3α	CTCCCTGCATACGCCAAA	AACAGAGTCGGTAGATGCG
sPLA2	GCCTGATCTTTCCCCAACACT	CTACGCAGCAGGAAGTTGGAT
DMBT1	AACTGGTGGTTTGTCGAGCAT	CCTACATCCCTCTTGGACCTTACTAC

[1] Abbreviations: Platelet phospholipase A2 (sPLA2) and deleted in malignant brain tumors 1 (DMBT1).

College of Wisconsin, Milwaukee, Wisconsin; World Wide Web: http://rgd.mcw.edu/gatool/). When present, duplicate entries representing the same transcript were averaged following statistical analysis. All data are presented as relative mean fold-change differences standardized to normal mucosal samples.

D. RESULTS.

We identified 472 transcripts (307 up-regulated, 165 down-regulated) differentially expressed in AOM-induced colon cancers compared to normal-appearing mucosa ($P < 0.05$). Genes with known identity/function (n=389) were broadly grouped into one of fourteen categories and included:

1. Cell adhesion, cell communication
2. Cell cycle, cell growth & maintenance, apoptosis
3. Channel, transporter, carrier proteins
4. Cytoskeleton, microtubule processes, structural filaments
5. Electron transport, oxidoreductase, detoxification
6. Extracellular matrix, tissue regeneration, wound repair
7. Immune, defense, inflammatory response
8. Metabolism
9. Proteolysis and peptidolysis
10. RNA processing
11. Signal transduction
12. Transcription regulation, nucleic acid binding
13. Enzymes

14. Other

FIGURE 1 depicts the functional distribution of genes expressed as a percentage of total known transcripts (n=389). Genes involved in immune, defense, and inflammation (13%, n=50), other (15%, n=59), signal transduction (10%, n=40), metabolism (7.2%, n=28), cell cycle, cell growth & maintenance, apoptosis (8.2%, n=32), and channel, transporter, carrier proteins (8.5%, n=33) represented the majority of genes changed between tissue types (mucosa *vs.* tumors).

A select list of differentially expressed transcripts is presented in **TABLE 2,** and a full listing of the remaining transcripts can be found in the **APPENDICES** as **SUPPLEMENTARY TABLE 1**. As shown in **TABLE 2,** genes involved in inflammation, chemotaxis, immune responses, and extracellular matrix remodeling were highly represented in colon cancers. Among specific genes repressed in tumor tissue included metabolic enzymes (PEPCK, carbonic anhydrase) and ion transport/carrier proteins as well as putative tumor suppressor genes (*DMBT1, APC*).

Quantitative RT-PCR.

RT-PCR was used to confirm gene changes for sPLA2, NP defensin 3α, lysozyme, and deleted in malignant brain tumors1 (DMBT1) and results are presented in **FIGURE 2**. There was general consistency in relative fold-changes with RT-PCR compared to values obtained from microarrays.

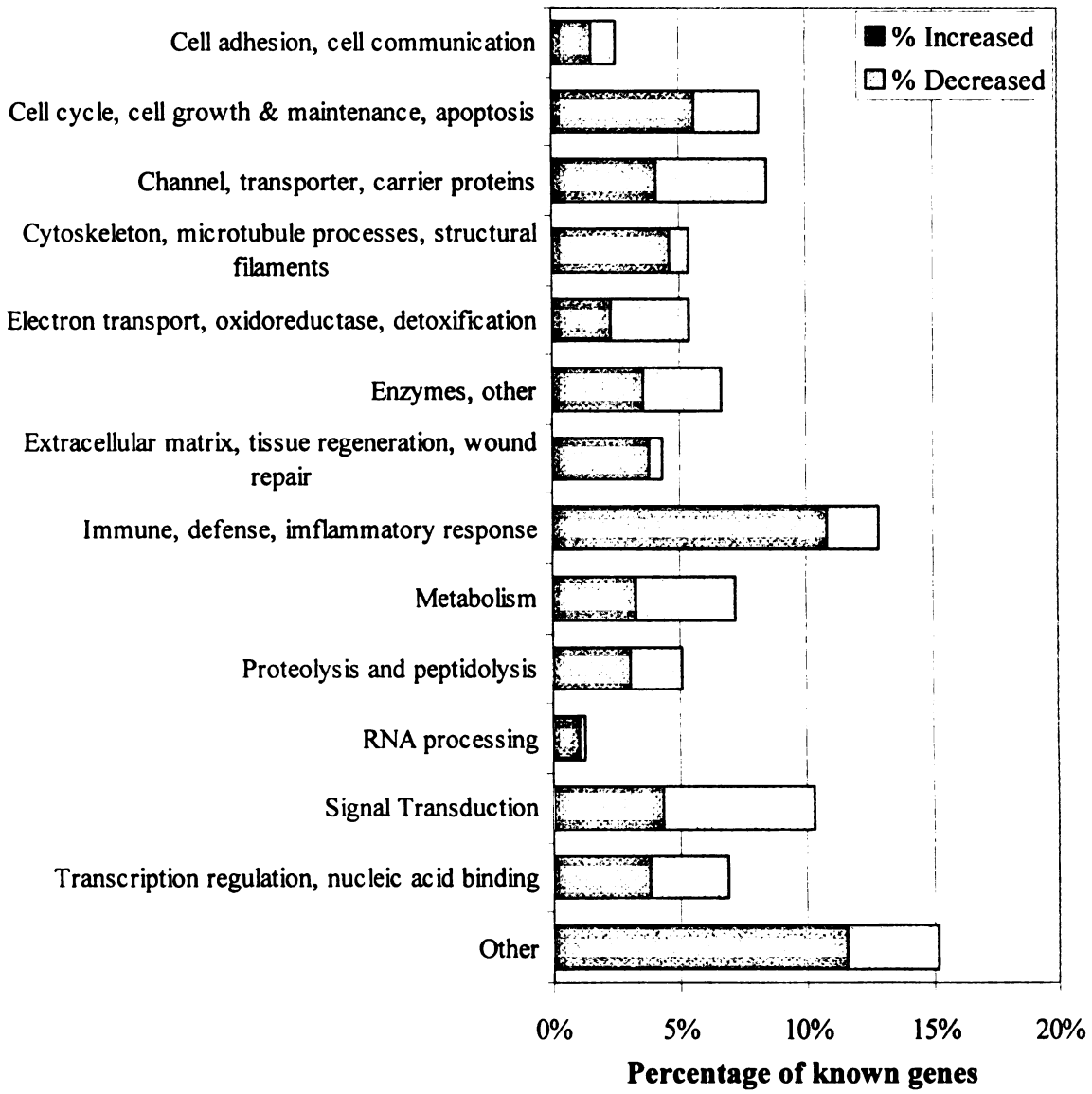


FIGURE 1. Biological classification of genes altered in AOM-induced colon cancers. Values expressed as a percentage based on the number of genes changed in colon cancer for each category divided by the total number of known genes (n=389).

TABLE 2. Genes significantly altered in AOM-induced colon cancers relative to normal colonic mucosa. [1]

Gene symbol	Gene Title	Tumor
I. Cell cycle, cell growth and maintenance, apoptosis		
Ptn	pleiotrophin	17
Igfbp3	insulin-like growth factor binding protein 3	13
---	lymphotoxin a	10
Sh3kbp1	sh3-domain kinase binding protein 1	10
Dab2	disabled homolog 2, mitogen-responsive phosphoprotein (drosophila)	6.7
---	v-myc avian myelocytomatosis viral oncogene homolog	4.5
Ripk3	receptor-interacting serine-threonine kinase 3	4.4
Fbln5	fibulin 5	3.7
Id2	inhibitor of dna binding 2, dominant negative helix-loop-helix protein	3.6
Napl1l	nucleosome assembly protein 1-like 1	3.2
Btg3	b-cell translocation gene 3	3.1
Tp53	tumor protein p53	3.0
Ngfrap1	nerve growth factor receptor associated protein 1	2.9
Psmd1	26s proteasome, subunit p112	2.8
Cdk4	cyclin-dependent kinase 4	2.6
Ns	nucleostemin	2.4
Ets1	v-ets erythroblastosis virus e26 oncogene homolog 1 (avian)	2.3
Adprt	ADP-ribosyltransferase 1	2.2
Pcna	proliferating cell nuclear antigen	2.2
Fyn	fyn proto-oncogene	2.2
---	cyclin e	2.2
Ccnd1	cyclin D1	2.1
Casp1	caspase 1	0.5
Prkcd	protein kinase c, delta	0.5
Btg1	b-cell translocation gene 1	0.5
Apc	adenomatosis polyposis coli	0.5

TABLE 2. Genes significantly altered in AOM-induced colon cancers relative to normal colonic mucosa. *(continued)* [1]

Gene symbol	Gene Title	Tumor
I. Cell cycle, cell growth and maintenance, apoptosis		
LOC60380	growth and transformation-dependent protein	0.4
Ceacam1	carcinoembryonic antigen-related cell adhesion molecule 1	0.4
Txnip	upregulated by 1,25-dihydroxyvitamin d-3	0.4
Pdgfa	platelet derived growth factor, alpha	0.3
Pmp22	peripheral myelin protein 22	0.2
Cgref1	cell growth regulator with EF hand domain 1	0.1
II. Channel, transporters, & carriers		
Fabp5	fatty acid binding protein 5, epidermal	8.9
Slc22a1	solute carrier family 22 (organic cation transporter), member 1	7.8
Rbp1	retinol binding protein 1	7.6
Slc11a2	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2	6.3
Slc7a1	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	5.4
Slc25a4	solute carrier family 25 (mitochondrial adenine nucleotide translocator) member 4	4.6
---	apolipoprotein e	3.9
Aqp1	aquaporin 1	3.6
---	hemoglobin beta chain complex	3.0
---	hemoglobin, alpha 1	2.7
Atp2c1	atpase, ca++-sequestering	2.6
Abp1	amiloride binding protein 1	2.2
Slc12a2	solute carrier family 12, member 2	2.1
Clns1a	chloride channel, nucleotide-sensitive, 1A	2.1
Nup155	nucleoporin 155kd	2.0
---	transcobalamin 2	2.0
Atp1a1	atpase, na+k+ transporting, alpha 1	0.5
Slc1a1	solute carrier family 1, member 1	0.5
P2rx4	purinergic receptor p2x, ligand-gated ion channel, 4	0.5

TABLE 2. Genes significantly altered in AOM-induced colon cancers relative to normal colonic mucosa. *(continued)* [1]

Gene symbol	Gene Title	Tumor
II. Channel, transporters, & carriers		
---	similar to solute carrier family 37 (glycerol-3-phosphate transporter), member 1 (loc294321), mrna	0.5
Slc5a6	solute carrier family 5, member 6	0.5
---	sulfotransferase family, cytosolic, 1c, member 2	0.4
---	chloride channel 2	0.4
Slc6a8	choline transporter	0.4
Z49858	plasmolipin	0.4
Slc16a1	solute carrier family 16, member 1	0.4
Slc5a3	solute carrier family 5 (inositol transporters), member 3	0.4
Slc25a10	solute carrier family 25 (mitochondrial carrier; dicarboxylate transporter), member 10	0.4
---	atpase na+/k+ transporting beta 1 polypeptide	0.3
Atp2a3	atpase, ca++ transporting, ubiquitous	0.3
Fxyd4	fxyd domain-containing ion transport regulator 4	0.3
Slc13a2	solute carrier family 13, member 2	0.2
Atp12a	atpase, h+/k+ transporting, nongastric, alpha polypeptide	0.1
III. Energy metabolism		
Ass	arginosuccinate synthetase	97
Arg1	arginase 1	17
Hsd11b1	hydroxysteroid 11-beta dehydrogenase 1	3.8
Me1	malic enzyme 1	3.5
Psat1	phosphoserine aminotransferase 1	3.4
Scd2	stearoyl-coenzyme a desaturase 2	2.6
Oat	ornithine aminotransferase	2.5
Pklr	pyruvate kinase, liver and rbc	2.3
---	phosphofructokinase, platelet	2.2
Fdps	farensyl diphosphate synthase	2.2
Acsl4	fatty acid coenzyme a ligase, long chain 4	2.1
Gnpat	acyl-coa:dihydroxyacetonephosphate acyltransferase	2.0

TABLE 2. Genes significantly altered in AOM-induced colon cancers relative to normal colonic mucosa. *(continued)* [1]

Gene symbol	Gene Title	Tumor
III. Energy metabolism		
Gyg	glycogenin	2.0
Glul	glutamine synthetase 1	0.5
Pcca	propionyl-coenzyme a carboxylase, alpha polypeptide	0.5
Acadm	acetyl-coenzyme a dehydrogenase, medium chain	0.5
Pygb	brain glycogen phosphorylase	0.5
Acads	short chain acyl-coenzyme a dehydrogenase	0.4
Sord	sorbitol dehydrogenase	0.4
Hk1	hexokinase 1	0.4
Gpt	glutamic-pyruvate transaminase (alanine aminotransferase)	0.4
---	Peptide YY	0.3
---	aldolase B	0.3
Hmgcs2	3-hydroxy-3-methylglutaryl-coenzyme a synthase 2	0.3
Eno3	enolase 3, beta	0.3
Pc	pyruvate carboxylase	0.2
Hpgd	nad-dependent 15-hydroxyprostaglandin dehydrogenase	0.1
---	phosphoenolpyruvate carboxykinase 1	0.0
IV. Extracellular matrix, tissur regeneration, wound repair		
Dcn	decorin	21
Apod	apolipoprotein d	14
Lum	lumican	12
Col5a2	collagen, type V, alpha 2	9.9
Sparc	secreted acidic cysteine rich glycoprotein	9.4
---	fibronectin 1	8.5
Col3a1	collagen, type iii, alpha 1	5.0
Col1a1	collagen, type 1, alpha 1	4.5
Col12a1	procollagen, type xii, alpha 1	4.1
Gpc2	glypican 2 (cerebroglycan)	3.9
Cyr61	cysteine rich protein 61	3.7

TABLE 2. Genes significantly altered in AOM-induced colon cancers relative to normal colonic mucosa. *(continued)* [1]

Gene symbol	Gene Title	Tumor
IV. Extracellular matrix, tissur regeneration, wound repair		
Pgsg	proteoglycan peptide core protein	3.6
Plod2	procollagen lysine, 2-oxoglutarate 5-dioxygenase 2	3.6
Lamc1	laminin, gamma 1	2.1
Cd81	CD 81 antigen	2.0
Tff3	trefoil factor 3	0.2
Dmbt1	deleted in malignant brain tumors 1	0.1
V. Immune, defense, imflammatory response		
---	rat anti-acetylcholine receptor antibody gene, rearranged ig gamma-2a chain, vdjc region, complete cds	207
---	immunoglobulin heavy chain 1a (serum IgG2a) (predicted)	37
---	defensin ratnp-3 precursor mrna, complete cds	29
---	gro	27
S100a9	s100 calcium-binding protein a9 (calgranulin b)	26
S100a8	s100 calcium-binding protein a8 (calgranulin a)	24
Fcgr3	Fc receptor, IgG, low affinity III	19
---	anti-ngf30 antibody light-chain	16
Tac1	tachykinin 1	15
Igha	partial mrna for immunoglobulin alpha heavy chain (partial), complete constant region	13
LOC60665	CXC chemokine LIX	12
C1s	complement component 1, s subcomponent	11
---	similar to complement component 1, r subcomponent (loc312705), mrna	11
Il1b	interleukin 1 beta	10
F2r	coagulation factor II (thrombin) receptor	8.4
Il1a	interleukin 1 alpha	8.0
Aif1	allograft inflammatory factor 1	7.2
---	MHC class II-associated invariant chain	6.9
C1qb	complement component 1, q subcomponent, beta polypeptide	6.2

TABLE 2. Genes significantly altered in AOM-induced colon cancers relative to normal colonic mucosa. *(continued)* [1]

Gene symbol	Gene Title	Tumor
V. Immune, defense, imflammatory response		
Nos2	nitric oxide synthase 2, inducible	6.1
Cxcl10	chemokine (C-X-C motif) ligand 10	6.1
Pla2g2a	phospholipase a2, group iia (platelets, synovial fluid)	6.1
---	ig germline kappa-chain c-region	6.1
F3	coagulation factor 3	5.8
Cd2	CD2 antigen	5.8
Ccl20	small inducible cytokine subfamily a20	5.0
Lyz	lysozyme	4.3
RT1-Db1	RT1 class II, locus Db1	3.9
Fcgr2b	fc receptor, igg, low affinity iib	3.8
---	chemokine (c-c motif) ligand 2	3.8
RT1-Bb	RT1 class II, locus Bb	3.3
---	prostaglandin-endoperoxide synthase 2	3.2
Cxcr4	chemokine (C-X-C motif) receptor 4	3.1
---	RT1 class II, locus Ba	2.8
Mfge8	milk fat globule-egf factor 8 protein	2.7
Mx1	myxovirus (influenza virus) resistance	2.6
Cd74	CD74 antigen (invariant polpypeptide of major histocompatibility class II antigen-associated)	2.6
Il6r	interleukin 6 receptor	2.5
RT1-Da	RT1 class II, locus Da	2.3
Il1r2	interleukin 1 receptor, type ii	2.2
Hla-dmb	rt1 class ii, locus dmb	2.0
Hla-dma	rt1 class ii, locus dma	2.0
Cd59	CD59 antigen	0.5
Cd14	CD14 antigen	0.5
Fcgrt	Fc receptor, IgG, alpha chain transporter	0.5
Tlr4	toll-like receptor 4	0.5

TABLE 2. Genes significantly altered in AOM-induced colon cancers relative to normal colonic mucosa. *(continued)* [1]

Gene symbol	Gene Title	Tumor
V. Immune, defense, imflammatory response		
---	polymeric immunoglobulin receptor	0.3
Il15	interleukin 15	0.3
Il18	interleukin 18	0.2
Sftpd	surfactant associated protein d	0.2
VI. Proteolysis and peptidolysis		
Mmp7	matrix metalloproteinase 7	46
Plat	plasminogen activator, tissue	19
Mmp12	matrix metalloproteinase 12	14
Mmp2	matrix metalloproteinase 2 (72 kda type iv collagenase)	13
Mmp13	matrix metalloproteinase 13	12
Pace4	subtilisin - like endoprotease	9.1
Plau	plasminogen activator, urokinase	4.4
Mmp3	matrix metalloproteinase 3	2.9
Ctsl	cathepsin L	2.3
Ctse	cathepsin E	2.3
Tpp2	tripeptidylpeptidase ii	2.1
Psmc2	proteasome (prosome, macropain) 26s subunit, atpase 2	2.0
Ctss	cathepsin S	0.5
Pcsk7	proprotein convertase subtilisin / kexin, type 7	0.5
Ctsd	cathepsin D	0.4
Klks3	kallikrein, submaxillary gland s3	0.4
Dpp4	dipeptidylpeptidase 4	0.3
Naaladl1	n-acetylated alpha-linked acidic dipeptidase-like (ileal peptidase i100)	0.2
Ngfg	nerve growth factor, gamma	0.2
Mep1a	meprin 1 alpha	0.2

[1] Data expressed as mean-fold change differences in tumor tissue relative to values for non-neoplastic colon mucosa (n = 2-4/group). A full listing of remaining transcripts can be found in Appendix A.

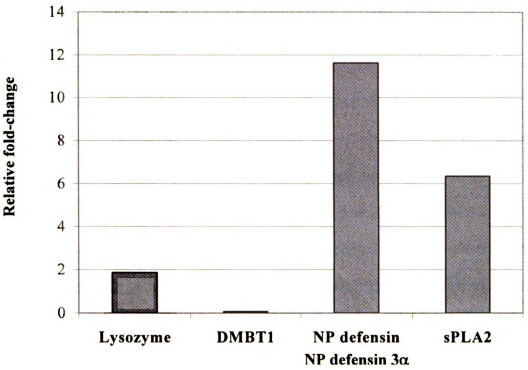


FIGURE 2. RT-PCR analysis of genes differentially expressed in AOM-induced colon cancers. Values represent relative fold-changes in RNA of tumor tissue normalized to non-neoplastic mucosa. *Abbreviations:* Deleted in malignant brain tumors 1 (*DMBT1*), group IIA secretory phospholipase A2 (sPLA2).

E. DISCUSSION.

Chronic inflammation has long been recognized to be involved in the etiology of colon cancer, and clinical, epidemiological, and experimental studies have established the efficacy of non-steroidal anti-inflammatory drugs (NSAIDs) in reducing the occurrence and mortality from CRC (9, 23). In this study, the most highly induced class of genes affected in colon tumors were those involved in inflammation and immunity with evidence of infiltration of lymphocytes, macrophages, neutrophils along with tissue damage and attempts at repair. For example up-regulation of COX-2, sPLA2, and iNOS, have been described previously in carcinogen-induced cancers as well as in human cancers (20-22, 24-25). The two former produce lipid mediators that stimulate cell proliferation and angiogenesis and inhibit apoptosis (26-29) whereas nitric oxide has been implicated in DNA damage and inactivation of tumor suppressor proteins through post-translational modification (30). S100 A and B are of macrophage and neutrophil origin and serum levels increase acutely during inflammation (31). S100 proteins form a lipid-scavenging complex within the cytosol of leukocytes and are thought to be a latent source of arachidonic acid for target cells at sites of inflammation (31). Also highly induced in AOM-induced colon cancers was the innate defense gene NP defensin 3α. NP defensin 3α is a cationic peptide induced during inflammation that exhibits a range of anti-microbial activity *in vitro (32)*. Melle *et al.* (33) recently reported potential usefulness of NP defensin 3α as a prognostic marker for recognition of individuals with colorectal cancer with a serum protein sensitivity of 69% and specificity of 100%.

In contrast to the number of inflammatory genes induced in colon cancers, we detected a lower expression of IL-18 and IL-15. IL-18 induces release of the Th1

cytokine interferon (IFN)-γ, which facilitates tumor cell recognition by stimulating cytotoxicity of natural killer (NK) cells, T lymphocytes, and macrophages and enhancing major histocompatability class (MHC) expression and antigen processing (34-35). IL-18 mRNA is depressed in human colon cancer tissue and absence of IL-18 is associated with poor prognosis including lymph node and liver metastasis (35). Similarly, IL-15 augments innate and adaptive immune responses by activating NK cell activity and stimulating CD8+ T cell recognition of tumor cells (36). A lower expression of IL-18 and IL-15 in AOM-induced tumors is suggestive of host evasion from immune surveillance and potential for metastatic capability.

Alterations in extracellular matrix (ECM) components are commonly observed during colon carcinogenesis and serve as a reservoir for growth factors, facilitate migration and proliferation, as well as contribute to angiogenesis (37). In this study AOM-induced colon cancers displayed higher transcripts for the ECM components decorin, fibronectin 1, and collagen 1α1. Decorin is a proteoglycan characteristic of stromal tissue (37) that is transcriptionally activated by hypomethylation in human colon cancer tissue (38). Decorin binds to and can inhibit transforming growth factor β (TGFβ), which normally exerts inhibitory effects on colonocyte proliferation (39). Fibronectin and collagen 1α1 have been implicated in a variety of processes including cell adhesion, migration, and chemotaxis. Both genes are increased in colon cancers and other hyperproliferative orders, and epithelial transcription decreases during terminal differentiation (40-43). Pleiotrophin, a heparin-binding cytokine was also highly expressed in AOM-induced colon cancer (44). The gene encoding pleiotrophin is

increased at active sites of neovasculargenesis including during development, ischemia, and in several cancers (44).

A high percentage of genes involved in the cell cycle were also affected in AOM-induced cancers. For example, tumors displayed a lower expression of the tumor suppressor gene *APC* and higher expression of the cell cycle regulators CDK4, cyclin E, and cyclin D1. *APC* mutations are common and permissive for human colon cancer development, but not frequently observed in AOM-induced colon cancers (17). APC expression increases in normal colon tissue as cells migrate up the colon crypt, and introduction of APC into cancer cells initiates apoptosis (45). Lower expression of *APC* in this study is likely a result of altered differentiation in colon tumors concomitant with evasion of apoptosis. AOM-induced tumors also displayed a lower expression of the glycoproteins CEACAM 1 and DMBT1. CEACAM1 was identified as a putative tumor suppressor gene involved in inducing apoptosis in response to hyperplasia (46). Nikkita *et al.* (46) reported loss of CEACAM 1 is occurs with a similar frequency in hyperplastic polyps and colon cancers, suggesting it precedes defects in the *APC* pathway. Loss of DMBT1 has not been previously reported in AOM colon cancers, but has been detected in human colon (47), neuroblastomas (48), and lung carcinomas (49), suggesting a possible tumor suppressive role. The function of DMBT1 has not been fully established, but has been implicated as a scavenger receptor involved in epithelial defense against bacteria (50).

Among genes most underrepresented in AOM-induced colon cancers included ion transporters and metabolic enzymes. The almost complete absence of some of the genes encoding enzymes involved in fatty acid oxidation and electron transport as well as in

gluconeogenesis, implies generalized defects in mitochondrial function and energy metabolism. Normal colon tissue preferentially oxidizes the short-chain fatty acid butyrate as an energy source (51). During cancer progression, a switch to glycolytic metabolism has been reported and thought to be a consequence of hypoxia in tumor tissue (52). A lower abundance of mitochondrial enzymes also been recently described in human cancers (53), and may explain the positive association of insulin resistance and hyperglycemia on CRC development, more so for advanced stages (54-56). Other metabolic enzymes including arginosuccinate synthase and arginase as well as cationic amino acid transporters were highly induced in colon cancers, potentially contributing to epithelial production of substrates for nitric oxide and polyamine synthesis, respectively.

In conclusion, this research provided more insight into global gene alterations contributing to AOM-induced colon carcinogenesis. A majority of genes induced during cancer progression are related to inflammatory and immune responses and angiogenesis with evidence of lower production of cytokines involved in anti-tumor immunity. Genes involved in extracellular matrix formation were also highly represented. A lower expression of ion transporters and metabolic enzymes highlights cellular disturbances in ion and water absorption and mitochondrial metabolism during cancer progression.

F. LITERATURE CITED.

1. Fearon ER and Vogelstein B. (1990) A Genetic Model for Colorectal Tumorigenesis. *Cell.* 61(5): 759-767.

2. Kinzler KW and Vogelstein B. (1996) Lessons from hereditary colorectal cancer. *Cell.* 87(2): 159-170.

3. Ilyas M, Straub J, Tomlinson IPM and Bodmer WF. (1999) Genetic pathways in colorectal and other cancers. *European Journal of Cancer.* 35(3): 335-351.

4. Williams NS, Gaynor RB, Scoggin S *et al.* (2003) Identification and validation of genes involved in the pathogenesis of colorectal cancer using cDNA microarrays and RNA interference. *Clinical Cancer Research.* 9(3): 931-946.

5. Bertucci F, Salas S, Eysteries S *et al.* (2004) Gene expression profiling of colon cancer by DNA microarrays and correlation with histoclinical parameters. *Oncogene.* 23(7): 1377-1391.

6. Barrier A, Lemoine A, Boelle PY *et al.* (2005) Colon cancer prognosis prediction by gene expression profiling. *Oncogene.* 24(40): 6155-6164.

7. Shih W, Chetty R and Tsao MS. (2005) Expression profiling by microarrays in colorectal cancer (Review). *Oncology Reports.* 13(3): 517-524.

8. Rhodes JM and Campbell BJ. (2002) Inflammation and colorectal cancer: IBD-associated and sporadic cancer compared. *Trends in Molecular Medicine.* 8(1): 10-16.

9. Herendeen JM and Lindley C. (2003) Use of NSAIDs for the chemoprevention of colorectal cancer. *Annals of Pharmacotherapy.* 37(11): 1664-1674.

10. American Cancer Society. (2005) Cancer Facts & Figures 2005. http://www.cancer.org/downloads/STT/CAFF2005f4PWSecured.pdf.

11. Pories SE, Ramchurren N, Summerhayes I and Steele G. (1993) Animal models for colon carcinogenesis. *Archives of Surgery.* 128: 647-53.

12. Reddy BS. (2004) Studies with the azoxymethane-rat preclinical model for assessing colon tumor development and chemoprevention. Environmental and molecular mutagenesis. 44 (1): 26-35.

13. Druckrey H, Preussman R, Matzkies F and Ivankovic S. (1967) Erzeugung von Darmkrebs bei Ratten durch 1,2-dimethylhydrazin. *Naturewissenschaften.* 54: 285-286, 1967.

14. Druckrey H. (1968) Organotropic carcinogenesis and mechanisms of action of symmetrical dialkylhydrazines and azo-and azoalkanes. *Food Cosmet. Toxicol.* 6: 578-579.

15. Druckery H. (1970) Production of colonic carcinomas by 1,2-dialkylhydrazines and azoalkanes. *In* Carcinoma of the colon and antecedent epithelium, WJ Burdette, ed. Springfield, Charles C Thomas; pp. 267-79.

16. Takahashi M and Wakabayashi K. (2004) Gene mutations and altered gene expression in azoxymethane-induced colon carcinogenesis in rodents. *Cancer Science.* 95(6): 475-480.

17. Caderni G, De Filippo C, Luceri C *et al.* (1997) *Apc* mutations in aberrant crypt foci (ACF) and colonic tumors induced by azoxymethane in F344 rats. *Proc. Amer. Assoc. Cancer Res.* 38: 467.

18. Takahashi M, Fukuda K, Sugimura T and Wakabayashi K. (1998) Beta-catenin is frequently mutated and demonstrates altered cellular location in azoxymethane-induced rat colon tumors. *Cancer Research.* 58(1): 42-46.

19. Vivona AA, Shpitz B, Medline A *et al.* (1993) K-Ras Mutations in Aberrant Crypt Foci, Adenomas and Adenocarcinomas During Azoxymethane-Induced Colon Carcinogenesis. *Carcinogenesis.* 14(9): 1777-1781.

20. DuBois RN, Radhika A, Reddy BS and Entingh AJ. (1996) Increased cyclooxygenase-2 levels in carcinogen-induced rat colonic tumors. *Gastroenterology.* 110(4): 1259-1262.

21. Watanabe K, Kawamori T, Nakatsugi S and Wakabayashi K. (2000) COX-2 and iNOS, good targets for chemoprevention of colon cancer. *Biofactors.* 12(1-4): 129-133.

22. Ohta T, Takahashib M, and Ochiaic A. (2005) Increased protein expression of both inducible nitric oxide synthase and cyclooxygenase-2 in human colon cancers. *Cancer Letters.* 1-8 (epub ahead of print).

23. Reddy BS, Rao CV and Seibert K. (1996) Evaluation of cyclooxygenase-2 inhibitor for potential chemopreventive properties in colon carcinogenesis. *Cancer Research.* 56(20): 4566-4569.

24. Yamashita S, Ogawa M, Sakamoto K *et al.* (1994) Elevation of Serum Group-Ii Phospholipase A(2) Levels in Patients with Advanced Cancer. *Clinica Chimica Acta.* 228(2): 91-99.

25. Ikegami T, Matsuzaki Y, Shoda J *et al.* (1998) The chemopreventive role of ursodeoxycholic acid in azoxymethane-treated rats: suppressive effects on enhanced group II phospholipase A(2) expression in colonic tissue. *Cancer Letters.* 134(2): 129-139.

26. Bingham CO and Austen KF. (1999) Phospholipase A(2) enzymes in eicosanoid generation. *Proceedings of the Association of American Physicians.* 111(6): 516-524.

27. Ashktorab H, Dawkins FW, Mohamed R *et al.* (2005) Apoptosis induced by aspirin and 5-fluorouracil in human colonic adenocarcinoma cells. *Digestive Diseases and Sciences.* 50(6): 1025-1032.

28. Tarnawski AS and Jones MK. (2003) Inhibition of angiogenesis by NSAIDs: molecular mechanisms and clinical implications. *Journal of Molecular Medicine-Jmm.* 81(10): 627-636.

29. Williams CS, Mann M and DuBois RN. (1999) The role of cyclooxygenases in inflammation, cancer, and development. *Oncogene.* 18(55): 7908-7916.

30. Pavlick KP, Laroux S, Fuseler J *et al.* (2002) Role of reactive metabolites of oxygen and nitrogen in inflammatory bowel disease. *Free Radical Biology and Medicine.* 33(3): 311-22.

31. Nacken W, Roth J, Sorg C and Kerkhoff C. (2003) S100A9/S100A8: Myeloid Representatives of the S100 Protein Family as Prominent Players in Innate Immunity. *Microsc. Res. Tech.* 60: 569–580.

32. Ganz T and Lehrer RI. (1994) Defensins. *Current Opinion in Immunology.* 6(4): 584-589.

33. Melle C, Ernst G, Schimmel B *et al* (2005) Discovery and Identification of α-Defensins as Low Abundant, Tumor-Derived Serum Markers in Colorectal Cancer. *Gastroenterology.* 129 (1): 66-73.

34. Tartour E and Fridman WH. (1998) Cytokines and cancer. *Int. Rev. Immunol.* 16: 683–704.

35. Pages F, Bergerb A, Lebel-Binaya S *et al.* (2000) Proinflammatory and antitumor properties of interleukin-18 in the gastrointestinal tract. *Immunology Letters.* 75(1): 9-14

36. Araki A, Hazama S, Yoshimura K *et al.* (2004) Tumor secreting high levels of IL-15 induces specific immunity to low immunogenic colon adenocarcinoma via CD8+ T cells. *Int J Mol Med.* 14(4): 571-6.

37. Wernert N. (1997) The multiple roles of tumour stroma. *Virchows Archiv-an International Journal of Pathology.* 430(6): 433-443.

38. Iozzo RV. (1995) Tumor Stroma as a Regulator of Neoplastic Behavior - Agonistic and Antagonistic Elements Embedded in the Same Connective-Tissue. *Laboratory Investigation.* 73(2): 157-160.

39. Massague J. (2000) How cells read TGF-beta signals. *Nature Reviews Molecular Cell Biology.* 1(3): 169-178.

40. Gradl D, Kuhl M and Wedlich D. (1999) The Wnt/Wg signal transducer beta-catenin controls fibronectin expression. *Molecular and Cellular Biology;* 19(8): 5576-5587.

41. Takayasu H, Horie H, Hiyama E *et al.* (2001) Frequent deletions and mutations of the beta-catenin gene are associated with overexpression of cyclin D1 and fibronectin and poorly differentiated histology in childhood hepatoblastoma. *Clinical Cancer Research.* 7(4): 901-908.

42. deGiorgio-Miller AM, Treharne LJ, McAnulty RJ *et al.* (2005) Procoltagen type I gene expression and cell proliferation are increased in lipodermatoscterosis. *British Journal of Dermatology.* 152(2): 242-249.

43. Vachon PH, Simoneau A, Herringgillam FE and Beaulieu JF. (1995) Cellular Fibronectin Expression Is Down-Regulated at the Messenger-RNA Level in Differentiating Human Intestinal Epithelial-Cells. *Experimental Cell Research.* 216(1): 30-34.

44. Christman KL, Fang Q, Kim AJ *et al.* (2005) Pleiotrophin induces formation of functional neovasculature in vivo. *Biochem Biophys Res Commun.* 332(4): 1146-52.

45. Morin PJ, Vogelstein B, Kinzler KW. (1996) Apoptosis and APC in colorectal tumorigenesis. *Proc Natl Acad Sci U S A.* 93(15):7950-4.

46. Nittka S, Gunther J, Ebisch C, *et al.* (2004) The human tumor suppressor CEACAM1 modulates apoptosis and is implicated in early colorectal tumorigenesis. *Oncogene.* 23(58): 9306-9313.

47. Mori M, Shiraishi T, Tanaka S *et al.* (1999) Lack of DMBT1 expression in oesophageal, gastric and colon cancers. *British Journal of Cancer.* 79(2): 211-213.

48. Munoz J, Lazcoz P, Inda MD *et al.* (2004) Homozygous deletion and expression of PTEN and DMBT1 in human primary neuroblastoma and cell lines. *International Journal of Cancer.* 109(5): 673-679.

49. Wu W, Kemp BL, Proctor ML *et al.* (1999) Expression of *DMBT1*, a Candidate Tumor Suppressor Gene, Is Frequently Lost in Lung Cancer. *Cancer Research.* 59(8): 1546-51.

50. Kang WQ and Reid KBM. (2003) DMBT1, a regulator of mucosal homeostasis through the linking of mucosal defense and regeneration? *Febs Letters.* 540(1-3): 21-25.

51. Roediger WE and Truelove SC. (1979) Method of preparing isolated colonic epithelial cells (colonocytes) for metabolic studies. *Gut.* 20(6): 484-8.

52. Dang CV and Semenza GL. (1999) Oncogenic alterations of metabolism. *Trends in Biochemical Sciences.* 24(2): 68-72.

53. Birkenkamp-Demtroder K, Christensen LL, Olesen SH *et al.* (2002) Gene expression in colorectal cancer. *Cancer Research.* 62: 4352-4363.

54. Chang CK and Ulrich CM. (2003) Hyperinsulinaemia and hyperglycaemia: possible risk factors of colorectal cancer among diabetic patients. *Diabetologia.* 46(5): 595-607.

55. Komninou D, Ayonote A, Richie JP and Rigas B. (2003) Insulin resistance and its contribution to colon carcinogenesis. *Experimental Biology and Medicine.* 228(4): 396-405.

56. Kaaks R, Toniolo P, Akhmedkhanov A *et al.* (2000) Serum C-peptide, insulin-like growth factor (IGF)-I, IGF-binding proteins, and colorectal cancer risk in women. *Journal of the National Cancer Institute.* 92(19): 1592-1600.

CHAPTER IV.

MICROARRAY ANALYSES OF GENES DIFFERENTIALLY EXPRESSED BY DIET (BLACK BEANS AND SOY FLOUR) DURING AOM-INDUCED COLON CARCINOGENESIS IN RATS

A. ABSTRACT.

We have previously demonstrated that feeding diets containing beans (black beans or soy flour) inhibits azoxymethane (AOM)-induced colon cancer in rats. The objective of this study was to identify genes altered during the early stages of AOM-induced colon carcinogenesis as well as candidate genes potentially involved in chemoprevention by beans. Ninety-five male Fischer F344 rats were obtained at 3 weeks of age and fed control (AIN-93G) diets upon arrival. At 4 and 5 weeks of age, rats were injected with AOM (15 mg/kg, n=75) or saline (SHAM, n=20) and at 6 weeks of age were separated into 3 groups and fed either the control (AIN), black bean (BB), or a soy flour (SF)-based diet. Rats were sacrificed at 36 weeks of age, and microarrays were performed on mRNA isolated from normal-appearing distal colonic mucosa. In animals treated with AOM, there was higher abundance of genes involved in immune function, including several MHC II-associated antigens and the antimicrobial genes lysozyme, NP defensin 3α, and sPLA2. BB- and SF-fed rats exhibited a higher expression of genes involved in energy metabolism and a lower expression of inflammatory (sPLA2, NP defensin 3α) and cell cycle-associated (cyclin B1, CDC2, TOP2A, GADD45α) genes. Genes involved in the extracellular matrix (collagen 1α1, fibronectin 1) and in innate immunity (NP defensin 3α, sPLA2) were induced by AOM injections in all diets, but were lowest in bean-fed animals. The gene change profile suggests that bean diets inhibit tumorigenesis by enhancing cellular differentiation and reducing early inflammatory events in non-neoplastic colonic mucosa.

B. INTRODUCTION.

Colorectal cancer (CRC) is currently the fourth most common cancer in the United States and the second leading cause of cancer mortality. An estimated 145, 290 new cases of CRC will be diagnosed alone in the US in 2005 (1). Although the development of CRC is likely multi-factorial, there is reason to believe that a majority of sporadic CRC cases can be substantially reduced, if not prevented, through modification of diet (2, 3). Among dietary factors associated with a decrease risk, epidemiological studies indicate that populations consuming higher intakes of legumes (peas, beans, lentils, peanuts) have a lower occurrence of (4-6) and mortality from CRC (7). Experiments conducted in our laboratory (8-12) and by others (13-14) have also demonstrated the potential of bean-based diets to inhibit carcinogen-induced colon cancer in rodents. For example, Hughes *et al.* (13) and Hangen and Bennink (12) found that rats fed dry beans (pinto, navy, or black beans) had a 50-57% lower incidence of colon cancer than rats fed a casein-based diet. Similarly, in a series of experiments, Bennink *et al.* (8-11) reported a significant reduction in colon tumor incidence and tumor burden in rats fed defatted soy flour (43%, 0.67 tumors/animal) compared to either ethanol-extracted soy concentrate (68%, 1.1 tumor/animal) or casein (68%, 1.43 tumor/animal). Several of the mechanisms proposed to account for the anti-tumorigenic effect of legumes have been attributed to the presence of one or more bioactive compounds (*ie.* phytoestrogens, protease inhibitors, phytic acid, saponins, and phytosterols), some of which inhibit one or more steps involved in cancer development (15-27). Although important, there is currently insufficient understanding of the cellular and molecular basis underlying colon

cancer inhibition by beans and the relative contribution of some of these compounds to the observed anti-neoplastic effects *in vivo*.

The azoxymethane (AOM)-treated rodent model has been utilized extensively to examine dietary influences on colon cancer, and many of the common genetic and pathogenic changes contributing to human colon cancers have also been observed during AOM-induced colon carcinogenesis (28-32). The purpose of this study was to provide insight into the cellular mechanisms contributing to colon cancer inhibition by beans *in vivo* by profiling changes in gene expression. Microarrays were performed on mRNA isolated from distal colonic epithelium of saline and AOM-injected F344 rats fed either a casein (AIN), black bean (BB), or defatted soy flour (SF) diet for 31 weeks. We chose to focus on the distal segment because most tumors develop in this area using standard protocol (15 mg/kg AOM) and there is evidence for site-specific effects of food constituents on tumorigenesis (11, 33). It was anticipated that genes most important to dietary suppression of tumorigenesis would be similarly affected by black beans and soy flour and have altered expression (increased or decreased) that corroborated tumor incidence.

C. MATERIALS AND METHODS.

Animal care and experimental diets.

This study was conducted in conformity with the regulatory guidelines of the Michigan State University Committee on Animal Use and Care. Ninety-five male Fischer (F344) rats were obtained from Harlan Sprague-Dawley (Indianapolis, IL) at 3 weeks of age and housed in plastic cages (2-3 rats/cage) in temperature (23°C ± 2°) and

humidity (40-60%) controlled rooms with a 12 hour light/dark cycle. Throughout the experiment animals had free access to food and distilled water and were assessed daily for health status and monthly for weight gain.

One control and two experimental diets were formulated based on the AIN-93G rodent diet with modifications (34), to contain the majority (ie >85%) of protein from either 1) high nitrogen casein (AIN), 2) black beans (BB), or 3) defatted soy flour (SF) (Archer Daniels Midland; Decantur, IL) and matched to have similar nutrient:energy ratios (**TABLE 1**). Black beans were soaked overnight in distilled water, conventionally cooked for 20-30 minutes, dried at 58 °C, and then finely ground through a 1.6 mm diameter mesh sieve prior to mixing with other diet ingredients. All diets contained approximately 18.9% (wt/wt) total protein, 11.3% dietary fiber, and 16.7% fat (wt/wt). Casein and tryptophan were added to black bean diet and methionine was added to all diets to increase the amino acid score. Lard and soybean oil were used as the fatty acid source, adjusted so the PUFA:MUFA:SFA ratios were 0.86:1.0:0.80 respectively.

Experimental design.

The design for this experiment was a 3X2 factorial with 3 dietary treatments (AIN, BB, SF) and 2 injection regimes (AOM, SHAM). Upon arrival, animals were fed the control (AIN) diet and allowed one week to acclimatize to new conditions. At 4 and 5 weeks of age, rats received subcutaneous injections (100 μL) of either 15 mg/kg of azoxymethane (AOM) prepared in saline (n=75; Ash Stevens, Detroit, MI) or saline (SHAM, n=20). Animals were fed the control (AIN) diet until one week after the final

TABLE 1. Nutrient composition of experimental diets.

	g/100g diet		
Ingredient	**AIN**	**Black Bean**	**Soy**
Casein	20	2.7	--
Dried, ground black beans	--	74	--
Defatted soy flour (55% protein)	--	--	34
Cornstarch	45	--	36
Sucrose	1.8	1.8	1.8
(Oil in diet)	(--)	(1.1)	(0.27)
Added soy oil	5.2	6.6	6.6
Corn oil	1.7	--	--
Lard	9.7	9.1	9.8
(Fiber in diet)	(--)	(11)	(5.0)
Added fiber (cellulose)	11	--	5.9
Mineral mix	3.9	3.9	3.9
Vitamin mix	1.1	1.1	1.1
Methionine	0.33	0.40	0.33
Tryptophan	--	0.004	--
Calcium carbonate	0.25	0.25	0.25
Choline bitartrate	0.28	0.28	0.28
Tert-butylhydroquinone	0.002	0.002	0.002
Totals:	**100**	**100**	**100**

injection, when they were randomized by weight to either continue on the control (AIN) diet or to be fed one of the experimental diets (BB or SF). At 36 weeks of age, animals were sacrificed by CO_2 inhalation and exsanguination, and the colon was immediately excised, opened longitudinally, and rinsed briefly in tap water to remove debris. Macroscopic tumors, when present, were excised and stored at –80 °C to be analyzed as a separate part of this study (**CHAPTER III**). The colon was then transected into proximal and distal segments and epithelial cells were collected by gently scraping normal-appearing mucosa from the distal half of the colon (excluding the lowermost 1 cm) with a glass slide. Samples were snap frozen and stored at –80 °C until RNA extraction could be performed.

Microarray target preparation and hybridization.

Affymetrix RU34A rat genome chips (Santa Clara, CA) were used in this experiment. For total RNA isolation, < 100 mg distal colonic mucosa was homogenized using a Tekmar homogenizer in one mL of TRIzol reagent containing 2 uL of RNase-free glycogen according to the manufacturers instructions (Gibco, Carlsbad, CA). After RNA extraction, samples were cleaned with RNeasy mini columns (Qiagen, Valencia, CA), quantified using a UV spectrophotometer (A_{260}/A_{280}), and the quality of RNA assessed by agarose-formaldehyde gel electrophoresis. Only high quality RNA was used in subsequent steps.

Biotinylated cRNA was prepared in accordance with instructions supplied in the GeneChip Expression Manual (Affymetrix, Santa Clara, CA). Double-stranded cDNA was synthesized from 10 μg of total RNA, pooled from 2-4 animals/treatment, using T7-$(dT)_{24}$ primers containing a T7 RNA polymerase promoter site (Proligo, Boulder, CO)

and the Superscript II system (Invitrogen, Carlsbad, California). Biotinylated cRNA was prepared using the Enzo BioArray HighYield RNA Transcript Labeling Kit (Affymetrix, Santa Clara, CA) and then purified with RNeasy mini columns. Approximately 15 μg cRNA was fragmented at 94 °C for 35 minutes and hybridized to RGU34A rat genome chips for 16 hours at 45 °C. Following hybridization, arrays were washed and stained with a streptavidin-phycoerythrin conjugate on an Affymetrix Fluidics station according to standard protocol. Processed arrays were scanned at 570 nm using a Hewlett Packard GeneArray Scanner.

Quantitative reverse transcription-PCR (RT-PCR) analysis.

The pooled RNA samples used for oligonucleotide microarrays were also used to confirm gene changes with RT-PCR. Gene specific primers for cell division cycle 2 (CDC2), cyclin B1, topoisomerase 2A (TOP2A), growth arrest and DNA-damage induced 45α (GADD45α), and group IIa secretory phospholipase A2 (sPLA2) were designed with the Primer Express 2.0 program (Applied Biosystems, Foster City, CA). The sequences of the primer pairs used are shown in **TABLE 2**. Single-stranded (ss) cDNA was synthesized from 2.5 μg of total RNA using T7-$(dT)_{24}$ primers (Proligo, Boulder, CO) and the Superscript II system (Invitrogen, Carlsbad, CA). Reverse transcription was performed in a thermocycler following the Superscript first strand synthesis protocol (Invitrogen, Carlsbad, California) with the following modifications: temperature of reaction was cooled to 20 °C for 5 minutes following annealing of primer and ss cDNA synthesis time was increased to 60 minutes for 42 °C. Following the reaction, ss cDNA was isolated with phase lock gel (Eppendorf), precipitated overnight, and the pellet was resuspended in ddH_2O to a working concentration of 10 ng/μL.

TABLE 2. Primer pairs used for RT-PCR[1]

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
β-Actin	AACCGTGAAAAGATGACCCAGAT	CACAGCCTGGATGGCTACGT
CDC2	GCTTGGACTTGCTCTCGAAAA	ATGGGTGCTTAAGGGCCATT
Cyclin B1	TGTGCACCTGCCGAAGAA	ACCATCGTCTGCATCTACATTCATC
TOP2A	GTCCGTTGAAAGAATCTATCAGAAAA	CCACAGAGCCGATGTAGGTATCT
GADD45α	GCTGGCTGCGGATGAAGAT	CACGAATGAGGGTGAAATGGAT
sPLA2	GCCTGATCTTTCCCCAACACT	CTACGCAGCAGGAAGTTGGAT

[1] Abbreviations: Cell division cycle 2 (CDC2), topoisomerase 2A (TOP2A), growth arrest and DNA-damage induced 45α (GADD45α), and group IIA secretory phospholipase A2 (sPLA2).

Quantitative determination of gene expression was performed with the ABI Prism7000 (Perkin Elmer Corp., Foster City, CA) using the Syber Green Universal Master Mix (Applied Biosystems, Foster City, CA). The reaction mixture (25 μL total volume) contained 20 ng ss cDNA, 12.5 μL SYBR Green RT-PCR Master mix, and 10.5 μL of diluted primers (forward and reverse). The final concentration (per 25 μL reaction volume) of primers was as follows: 600 nM for TOP2A and cyclin B1, and 1.2 μM for $sPLA_2$, CDC2, GADD45α, and β-Actin. The real-time cycle conditions were as follows: PCR initial activation step at 95 °C for 15 min and a total of 40 cycles for melting (95 °C, 15 s) and annealing/extension (60 °C, 1 min). All assays were performed in duplicates, using 2-3 samples per group and relative fold-changes were quantified by using the comparative CT ($\Delta\Delta C_T$) method (Applied Biosystems, User Bulletin #2) with the AIN(saline-injected) group as the calibrator.

Statistical analysis methods.

Data for weight gain, microarrays, and RT-PCR were analyzed using the General Linear Models procedure of SAS (SAS Institute, Cary, NC, Version 7.0). When statistical differences were detected with the F statistic, individual comparisons were made using the least significant difference (LSD) method. Tumor incidence data was analyzed with a χ^2 test using the Proc Freq procedure in SAS.

Prior to statistical analyses fluorescence intensity data from microarrays were globally scaled to a target intensity of 500 in Affymetrix Microarray Suite, version 5.0 to control within-chip variations. Globally scaled data were then imported into GeneSpring (Silicon Genetics, Inc., Redwood City, CA, Version 6.0) for normalization and filtering. All chips were normalized to the median intensity of a set of invariant genes whose

expression across all conditions (injection type, diet) after global scaling showed less than a 30% coefficient of variation (CV). A filtering step excluded genes not considered "Present" or "Marginal" in at least 42% (10 of 24) of the samples. This step reduced the number of genes to be analyzed from 8799 to 3931 genes (45% of genes present on the chip). An additional filtering step limited the genes further to those exhibiting greater than 1.3 or less than 0.7 fold-change difference between diets or injection type. Normalized expression values were analyzed using the GLM procedure of SAS. When present, duplicate transcripts were averaged following statistical analysis.

Differentially expressed transcripts ($P<0.05$) were broadly grouped into categories based on known gene ontologies and biological functions reported in the literature. Gene ontologies were retrieved using the Affymetrix NetAffix Analysis Center (http://www.affymetrix.com/analysis/index.affx) and the Rat Genome Database (RGD) gene annotation tool (Rat Genome Database Web Site, Medical College of Wisconsin, Milwaukee, Wisconsin; World Wide Web: http://rgd.mcw.edu/gatool/). For brevity, only transcripts with known identities are shown. Data are presented as mean fold-change differences standardized to the AIN (control) group for diet-dependent differences or to the saline-injected group for injection-dependent changes.

D. RESULTS.

Weight gain and tumor incidence.

There were no significant main effects of diet (AIN, BB, SF) or injection regime (AOM *vs* saline) on body weight gain. The total weight gain (g, LSM ± SEM) of rats while on experimental diets was: AIN = 304 ± 7.4, BB = 287 ± 8.3, SF = 299 ± 8.2.

There was a significant effect of diet on tumor incidence ($P = 0.03$). As previously established, bean-based diets inhibited tumor incidence by ~60% compared to rats fed the control diet. Tumor incidence was 75%, 33%, and 25% for rats fed the AIN, BB, or SF diets, respectively ($P < 0.05$).

Microarray analysis.

Among the 8799 genes and ESTs present on the rat genome UG34A array, 316 transcripts passed the multiple filtering criteria employed to be further analyzed for injection- and 928 for diet-dependent changes (see under statistical analyses). A total of 157 transcripts were significantly affected by injection regime (AOM *vs.* saline), 257 by dietary treatment (AIN, BB, SF), and 9 were common to both lists ($P < 0.05$). Transcripts differentially expressed were broadly classified into one of eleven functional categories (**FIGURE 1**) and included:

(1) Cell cycle, cell growth & maintenance, apoptosis

(2) Channel, transporter, carrier proteins

(3) Electron transfer, oxidoreductase, detoxification

(4) Energy metabolism

(5) Extracellular matrix, cell adhesion, cytoskeleton

(6) Immune, defense, stress

(7) Intracellular protein metabolism

(8) Nucleic acid binding, transcription regulation

(9) Signal transduction

(10) Enzymes, other

(11) Other

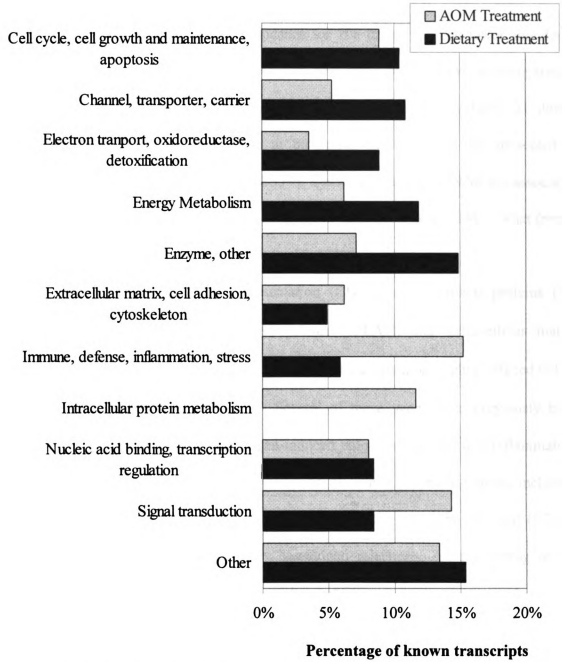


FIGURE 1. Biological classification of known transcripts significantly altered in the distal colonic epithelium of male F344 rats as affected by AOM-injections and dietary (AIN, BB, SF) treatment ($P < 0.05$).

Genes differentially expressed by carcinogen (AOM) injection.

There was a greater mRNA abundance for 108 transcripts and lower abundance for 48 transcripts in the colon of rats injected with carcinogen (AOM) as compared to saline-injected controls (SHAMs). Of these genes, 112 (75 up-regulated, 37 down-regulated) were categorized according to biological functions and are presented in **FIGURE 2**. As shown, a majority of known transcripts affected (59%) are associated with immune, defense, stress (n=17, 15%), signal transduction (n=16, 14%), other (n=15, 13%), or intracellular protein metabolism (n=13, 12%).

Genes involved in antigen presentation (CD74), anti-microbial proteins (NP defensin 3α, lysozyme), inflammatory proteins (sPLA2), and extracellular matrix proteins (collagen 1α1, fibronectin 1) were among the genes most highly induced (>1.5-fold) by AOM-treatment (**TABLE 3**). Several of these genes have previously been shown to be over-expressed in carcinogen-induced colon cancer (35) and inflammatory conditions of the colon (36). Genes involved in ribosomal protein synthesis, including components of the 40S ribosomal protein subunit (s7, s4, s9) and the 60S unit (l37, l4, l36a) were also moderately induced by carcinogen injections. A full listing of the remaining transcripts significantly affected by AOM treatment is available in the **APPENDICES** as **SUPPLEMENTARY TABLE 2.**

TABLE 3. Select genes differentially expressed by carcinogen (AOM) treatment in the distal colonic epithelium of male F344 rats. [1]

Gene symbol	Common	Saline-Injected	AOM-Injected
I. Extracellular matrix, cell adhesion, cytoskeleton			
Col1a1	collagen, type 1, alpha 1	1.0 [a]	1.8 [b]
Fn1	fibronectin 1	1.0 [a]	1.7 [b]
Vim	vimentin	1.0 [a]	1.5 [b]
Tmsb10	thymosin, beta 10	1.0 [a]	1.3 [b]
Col3a1	collagen, type iii, alpha 1	1.0 [a]	1.3 [b]
Ceacam10	c-cam4 protein	1.0 [a]	1.3 [b]
Cdc42bpb	cdc42-binding protein kinase beta	1.0 [b]	0.6 [a]
II. Immune, defense, inflammation, stress			
----	similar to IG kappa-chain V-V region K2 precursor	1.0 [a]	3.3 [b]
Pla2g2a	phospholipase a2, group iia	1.0 [a]	2.4 [b]
Lyz	lysozyme	1.0 [a]	2.1 [b]
RT1-Db1	rt1 class ii, locus db1	1.0 [a]	1.8 [b]
Cd74	cd74 antigen (invariant polpypeptide of major histocompatibility class ii antigen-associated)	1.0 [a]	1.8 [b]
----	Ig active lambda2-like chain	1.0 [a]	1.7 [b]
Hla-dmb	rt1 class ii, locus dmb	1.0 [a]	1.7 [b]
----	NP defensin 3 alpha precursor	1.0 [a]	1.7 [b]
RT1-Da	rt1 class ii, locus da	1.0 [a]	1.6 [b]
RT1-Ba	rt1 class ii, locus ba	1.0 [a]	1.5 [b]
RT1-Bb	rt1 class ii, locus bb	1.0 [a]	1.5 [b]
Ccl2	chemokine (c-c motif) ligand 2	1.0 [a]	1.4 [b]
Mif	macrophage migration inhibitory factor	1.0 [a]	1.3 [b]
Psmb9	proteosome (prosome, macropain) subunit, beta type 9	1.0 [a]	1.3 [b]
Mx1	myxovirus (influenza virus) resistance	1.0 [a]	1.3 [b]
RT1-M3	rt1 class ib, locus m3	1.0 [a]	1.3 [b]
Il15	interleukin 15	1.0 [b]	0.6 [a]

TABLE 3. Select genes differentially expressed by carcinogen (AOM) treatment in the distal colonic epithelium of male F344 rats. *(continued)* [1]

Gene symbol	Common	Saline-Injected	AOM-Injected
III. Intracellular protein metabolism			
Rps7	ribosomal protein s7	1.0 [a]	1.4 [b]
Rps15	ribosomal protein s15	1.0 [a]	1.4 [b]
Hspe1	heat shock 10 kda protein 1	1.0 [a]	1.3 [b]
Rpl37	ribosomal protein l37	1.0 [a]	1.3 [b]
Rpl4	ribosomal protein l4	1.0 [a]	1.3 [b]
Psmb4	proteasome (prosome, macropain) subunit, beta type 4	1.0 [a]	1.3 [b]
Rps4x	ribosomal protein s4, x-linked	1.0 [a]	1.3 [b]
Rps17	ribosomal protein s17	1.0 [a]	1.3 [b]
Rpl36a	large subunit ribosomal protein l36a	1.0 [a]	1.3 [b]
Rps9	ribosomal protein s9	1.0 [a]	1.3 [b]
Pcsk3	proprotein convertase subtilisin/kexin type3	1.0 [a]	1.3 [b]
Pam	peptidylglycine alpha-amidating monooxygenase	1.0 [b]	0.6 [a]
Rps24	ribosomal protein s24	1.0 [b]	0.6 [a]

[1] Data expressed as mean-fold change differences standardized to the SHAM (saline-injected) animals (n=12/group). Superscripts denote significant main effects for carcinogen treatment ($P < 0.05$).

Genes differentially expressed by dietary treatment.

Among known transcripts differentially affected by dietary treatment (n=202), compared to the AIN diet, feeding rats black beans (BB) significantly affected 145 genes (90 up-regulated, 55 down-regulated) and soy flour (SF) affected 115 genes (87 up-regulated, 28 down-regulated). Fifty genes were significantly co-induced and 22 co-repressed by BB and SF, representing 36% of gene changes, although an additional 30% showed the same direction of change. A majority of known transcripts affected by dietary treatment (54%) fell into one of five categories including other (n=31, 15%), enzymes (n=30, 15%), energy metabolism (n=24, 12%), channel, transporter, carrier proteins (n=22, 11%), and cell cycle and apoptosis (n=21, 10%) **(FIGURE 2).**

A select listing of transcripts similarly affected in the colon of bean-fed compared to casein-fed rats is presented in **TABLE 4**, and a full listing of the remaining transcripts can be found in the **APPENDICES** as **SUPPLEMENTARY TABLE 3**. As shown in **TABLE 4**, bean-based diets co-induced a number of genes involved in fatty acid metabolism and gluconeogenesis (HMG CoA synthase 2, PEPCK, enoyl CoA hydratase 1), electron transport (CYPp450 4b1&2d1, diaphorase 1), detoxification (gluthionine s transferase, UDP glycotransferases, perixoredoxin) and solute/ion transport (aquaporin 8, sodium channel, solute carriers). Among genes co-repressed included some cell cycle associated proteins (cyclin B1, CDC2), fatty acid desaturation enzymes (stearoyl Co-A desaturase 1&2), extracellular matrix proteins (collagen 1α, fibronectin 1), innate immune response/inflammatory proteins (MIF, sPLA2, NP defensin 3α, toll-like receptor 4, cd1d1 antigen), and proteins involved in nucleic acid binding/transcription regulation (high mobility group box 2, early growth response 1 & 2).

TABLE 4. Genes similarly affected by bean-feeding (BB and SF) in the distal colonic epithelium of male F344 rats. [1]

Gene symbol	Gene Title	AIN	BB	SF
I. Cell cycle, cell growth and maintenance, apoptosis				
Ceacam1	carcinoembryonic antigen-related cell adhesion molecule 1	1.0 [a]	2.3 [b]	1.9 [b]
Rb1	retinoblastoma 1	1.0 [a]	2.2 [b]	2.2 [b]
Sgk	serum/glucocorticoid regulated kinase	1.0 [a]	1.9 [b]	1.6 [b]
Gadd45a	growth arrest and dna-damage-inducible 45 alpha	1.0 [a]	1.5 [b]	1.5 [b]
Txnip	upregulated by 1,25-dihydroxyvitamin d-3	1.0 [a]	1.4 [b]	1.3 [b]
Csnk1d	casein kinase 1, delta	1.0 [a]	1.3 [b]	1.2 [b]
Bax	bcl2-associated x protein	1.0 [a]	1.3 [b]	1.4 [b]
Egln3	egl nine homolog 3 (c. elegans)	1.0 [b]	0.7 [a]	0.8 [ab]
Wfdc1	wap four-disulfide core domain 1	1.0 [b]	0.7 [a]	0.5 [a]
Ccnb1	cyclin b1	1.0 [b]	0.7 [a]	0.8 [ab]
Cdc2a	cell division cycle 2 homolog a (s. pombe)	1.0 [b]	0.6 [a]	0.8 [b]
Top2a	topoisomerase (DNA) 2 alpha	1.0 [b]	0.6 [a]	0.8 [b]
II. Channel, transporters, & carriers				
Aqp8	aquaporin 8	1.0 [a]	2.7 [b]	2.9 [b]
Scnn1g	sodium channel, nonvoltage-gated 1 gamma	1.0 [a]	2.2 [b]	2.0 [b]
---	apolipoprotein a-i	1.0 [a]	1.6 [ab]	2.4 [b]
---	k-cl cotransporter kcc4	1.0 [a]	1.6 [b]	1.4 [b]
Tfrc	transferrin receptor	1.0 [a]	1.5 [b]	1.2 [ab]
Slc5a1	solute carrier family 5, member 1	1.0 [a]	1.5 [b]	1.3 [ab]
Slc16a1	solute carrier family 16, member 1	1.0 [a]	1.5 [b]	1.5 [b]
Atp1a1	atpase, na+k+ transporting, alpha 1	1.0 [a]	1.4 [b]	1.3 [b]
Lgals9	lectin, galactose binding, soluble 9	1.0 [a]	1.3 [b]	1.3 [b]
Kcnk1	putative potassium channel twik	1.0 [a]	1.3 [b]	1.3 [b]
Slc16a6	solute carrier family 16 (monocarboxylic acid transporters), member 6	1.0 [b]	0.6 [a]	0.8 [b]
Fabp5	fatty acid binding protein 5, epidermal	1.0 [b]	0.5 [a]	0.6 [a]
---	tocopherol (alpha) transfer protein	1.0 [b]	0.5 [a]	0.7 [a]

TABLE 4. Genes similarly affected by bean-feeding (BB and Soy) in the distal colonic epithelium of male F344 rats. (*continued*) [1]

Gene symbol	Gene Title	AIN	BB	SF
III. Electron transport, oxidoreductase, detoxification				
Cyp4b1	cytochrome p450, subfamily 4b, polypeptide 1	1.0 [a]	3.6 [b]	2.2 [c]
---	udp glycosyltransferase 1 family, polypeptide a1	1.0 [a]	1.7 [b]	2.2 [b]
Gstm5	glutathione s-transferase, mu 5	1.0 [a]	1.5 [b]	1.5 [b]
Gstm1	glutathione s-transferase, mu 1	1.0 [a]	1.4 [b]	1.3 [ab]
Prdx6	peroxiredoxin 6	1.0 [a]	1.4 [ab]	1.5 [b]
Cyp2d10	cytochrome p450 2d1	1.0 [a]	1.4 [b]	1.4 [b]
Sult1a1	sulfotransferase family 1a, phenol-preferring, member 1	1.0 [a]	1.4 [b]	1.2 [ab]
Por	p450 (cytochrome) oxidoreductase	1.0 [a]	1.4 [b]	1.4 [b]
Cyb5	cytochrome b5	1.0 [a]	1.4 [b]	1.3 [b]
Etfdh	electron-transferring-flavoprotein dehydrogenase	1.0 [a]	1.4 [b]	1.2 [ab]
Tst	thiosulfate sulfurtransferase	1.0 [a]	1.3 [b]	1.3 [b]
Dia1	diaphorase 1	1.0 [a]	1.2 [ab]	1.3 [b]
Cbr1	carbonyl reductase 1	1.0 [a]	1.2 [ab]	1.5 [b]
---	udp glycosyltransferase 1 family, polypeptide a7	1.0 [a]	1.2 [b]	1.3 [b]
---	udp glycosyltransferase 1 family, polypeptide a6	1.0 [a]	1.2 [a]	1.5 [b]
IV. Energy metabolism				
Hmgcs2	3-hydroxy-3-methylglutaryl-coenzyme a synthase 2	1.0 [a]	2.2 [b]	2.1 [b]
Aldob	aldolase b	1.0 [a]	2.1 [b]	1.8 [b]
---	phosphoenolpyruvate carboxykinase 1	1.0 [a]	2.0 [b]	1.8 [b]
Ech1	enoyl coenzyme a hydratase 1	1.0 [a]	1.6 [b]	1.6 [b]
Glul	glutamine synthetase 1	1.0 [a]	1.4 [c]	1.2 [b]
Hadhb	hydroxyacyl-coenzyme a dehydrogenase/3-ketoacyl-coenzyme a thiolase/enoyl-coenzyme a hydratase (trifunctional protein), beta subunit	1.0 [a]	1.3 [b]	1.3 [b]
Hsd17b2	17-beta hydroxysteroid dehydrogenase type 2	1.0 [a]	1.2 [ab]	1.4 [b]
Sqle	squalene epoxidase	1.0 [a]	1.2 [ab]	1.3 [b]
Mte1	mitochondrial acyl-coa thioesterase 1	1.0 [a]	1.2 [ab]	1.8 [b]

TABLE 4. Genes similarly affected by bean-feeding (BB and Soy) in the distal colonic epithelium of male F344 rats. (*continued*) [1]

Gene symbol	Gene Title	AIN	BB	SF
IV. Energy metabolism				
Hadha	hydroxyacyl-coenzyme a dehydrogenase/3-ketoacyl-coenzyme a hiolase/enoyl-coenzyme a hydratase (trifunctional protein), alpha subunit	1.0 [a]	1.2 [b]	1.3 [b]
---	phosphofructokinase, platelet	1.0 [b]	0.7 [a]	0.8 [a]
Gpd2	glycerol-3-phosphate dehydrogenase 2	1.0 [b]	0.7 [a]	0.8 [ab]
Acsl4	fatty acid coenzyme a ligase, long chain 4	1.0 [b]	0.6 [a]	0.8 [ab]
---	peptide YY	1.0 [b]	0.6 [a]	0.7 [a]
---	glucagon	1.0 [b]	0.6 [a]	0.8 [ab]
Scd2	stearoyl-coenzyme a desaturase 2	1.0 [b]	0.5 [a]	0.7 [ab]
Scd1	stearoyl-coenzyme a desaturase 1	1.0 [b]	0.5 [a]	0.7 [ab]
V. Extracellular matrix, cell adhesion, cytoskeleton				
---	cd36 antigen-like 2	1.0 [a]	1.4 [b]	1.2 [ab]
Sdc1	syndecan 1	1.0 [a]	1.3 [b]	1.3 [b]
Fn1	fibronectin 1	1.0 [b]	0.7 [a]	0.6 [a]
Tubb5	tubulin, beta 5	1.0 [b]	0.6 [a]	0.9 [b]
Stmn1	stathmin 1	1.0 [b]	0.6 [a]	0.9 [ab]
Col1a1	collagen, type 1, alpha 1	1.0 [b]	0.5 [a]	0.5 [a]
VI. Immune, defense, inflammation, stress				
Hspa1a	heat shock 70kd protein 1a	1.0 [a]	1.9 [b]	1.4 [ab]
RT1-Aw2	rt1 class ib, locus aw2	1.0 [a]	1.5 [b]	1.4 [b]
Tff3	trefoil factor 3	1.0 [a]	1.5 [b]	1.2 [ab]
RT1-Aw2	mhc class i rt1.o type 149 processed pseudogene	1.0 [a]	1.3 [b]	1.2 [b]
---	protease (prosome, macropain) 28 subunit, beta	1.0 [a]	1.3 [b]	1.2 [ab]
Tlr4	toll-like receptor 4	1.0 [b]	0.8 [a]	0.7 [a]
Mif	macrophage migration inhibitory factor	1.0 [b]	0.7 [a]	0.9 [ab]
Dmbt1	deleted in malignant brain tumors 1	1.0 [b]	0.6 [a]	0.7 [a]
Pla2g2a	phospholipase a2, group iia	1.0 [b]	0.5 [a]	0.4 [a]
---	NP defensin 3 alpha precursor	1.0 [b]	0.5 [a]	0.7 [a]

TABLE 4. Genes similarly affected by bean-feeding (BB and Soy) in the distal colonic epithelium of male F344 rats. (*continued*) [1]

Gene symbol	Gene Title	AIN	BB	SF
VII. Nucleic acid binding, transcription regulation				
Nr1d2	nuclear receptor subfamily 1, group d, member 2	1.0 [a]	2.2 [b]	1.3 [a]
Pem	placentae and embryos oncofetal gene	1.0 [a]	1.6 [b]	1.3 [ab]
Nfib	nuclear factor i/b	1.0 [a]	1.5 [b]	1.4 [b]
Vdr	vitamin d receptor	1.0 [a]	1.4 [ab]	1.6 [b]
Pdx1	synaptotagmin 4	1.0 [b]	0.8 [ab]	0.6 [a]
Egr2	early growth response 2	1.0 [b]	0.8 [a]	0.6 [a]
Gata6	gata binding protein 6	1.0 [b]	0.7 [a]	0.9 [ab]
Nr4a2	nuclear receptor subfamily 4, group a, member 2	1.0 [b]	0.7 [a]	0.7 [a]
Hmgb2	high mobility group box 2	1.0 [b]	0.7 [a]	0.9 [b]
Egr1	early growth response 1	1.0 [b]	0.5 [a]	0.7 [a]
VIII. Signal transduction				
Guca2a	guanylate cyclase activator 2a	1.0 [a]	1.5 [b]	1.4 [ab]
Csrp2	cysteine-rich protein 2	1.0 [a]	1.5 [b]	1.5 [b]
Sstr2	somatostatin receptor 2	1.0 [a]	1.5 [b]	1.7 [b]
Mapk14	mitogen activated protein kinase 14	1.0 [a]	1.3 [b]	1.4 [b]
Gucy2c	guanylate cyclase 2c	1.0 [a]	1.3 [b]	1.4 [b]
P2ry2	purinergic receptor p2y, g-protein coupled 2	1.0 [b]	0.8 [ab]	0.6 [a]
P2ry6	pyrimidinergic receptor p2y, g-protein coupled, 6	1.0 [b]	0.7 [a]	0.7 [a]
Pld1	phospholipase d1	1.0 [b]	0.7 [a]	0.9 [b]
Ptpro	protein tyrosine phosphatase, receptor type o	1.0 [b]	0.6 [a]	0.7 [a]
---	frizzled homolog 1 (drosophila)	1.0 [b]	0.6 [a]	0.7 [a]
IX. Enzymes, other				
Plat	plasminogen activator, tissue	1.0 [a]	3.0 [b]	2.4 [ab]
Abat	4-aminobutyrate aminotransferase	1.0 [a]	3.0 [c]	1.7 [b]
Ca4	carbonic anhydrase 4	1.0 [a]	1.7 [b]	1.7 [b]
---	protein phosphatase 1b, magnesium dependent, beta isoform	1.0 [a]	1.6 [b]	1.4 [ab]
Ptpn1	protein tyrosine phosphatase, non-receptor type 1	1.0 [a]	1.6 [b]	1.3 [ab]

TABLE 4. Genes similarly affected by bean-feeding (BB and Soy) in the distal colonic epithelium of male F344 rats. (*continued*) [1]

Gene symbol	Gene Title	AIN	BB	SF
IX. Enzymes, other				
Oas1	25 oligoadenylate synthetase	1.0 [a]	1.5 [b]	1.5 [b]
Dpp4	dipeptidylpeptidase 4	1.0 [a]	1.4 [b]	1.3 [b]
Cyp27a1	cytochrome p450, family 27, subfamily a, polypeptide 1	1.0 [a]	1.4 [b]	1.5 [b]
Ache	acetylcholinesterase	1.0 [a]	1.4 [b]	1.5 [b]
Adk	adenosine kinase	1.0 [a]	1.3 [b]	1.5 [b]
Pts	6-pyruvoyl-tetrahydropterin synthase	1.0 [a]	1.3 [ab]	1.5 [b]
Gcnt3	beta-1,6-n-acetylglucosaminyltransferase	1.0 [b]	0.7 [a]	0.7 [a]
Ctse	cathepsin e	1.0 [b]	0.7 [a]	0.7 [a]
X. Other				
Map17	membrane-associated protein 17	1.0 [a]	2.7 [b]	1.4 [a]
Zg16	zymogen granule protein 16	1.0 [a]	2.2 [b]	1.9 [b]
Spink1	atpase inhibitor	1.0 [a]	2.1 [b]	1.4 [a]
---	germinal histone h4 gene	1.0 [a]	2.0 [b]	1.4 [ab]
Gchfr	gtp cyclohydrolase i feedback regulatory protein	1.0 [a]	1.9 [b]	1.8 [b]
---	calbindin 3	1.0 [a]	1.6 [b]	1.2 [a]
Muc1	mucin 1	1.0 [a]	1.6 [c]	1.3 [b]
Ensa	endosulfine alpha	1.0 [a]	1.5 [b]	1.4 [ab]
F3	coagulation factor 3	1.0 [b]	0.7 [a]	0.5 [a]
---	annexin 5	1.0 [c]	0.5 [a]	0.7 [b]
Bub1b	budding uninhibited by benzimidazoles 1 homolog, beta (s. cerevisiae)	1.0 [b]	0.5 [a]	0.6 [a]

[1] Data expressed as mean-fold change differences standardized to the AIN group (n=8/group). Superscripts denote significant main effects for diet ($P < 0.05$).

Genes affected by dietary treatment and carcinogen injection.

Nine genes were found to be influenced both by diet and carcinogen and are presented in **TABLE 5**. Transcripts for collagen 1α1, fibronectin 1, NP defensin 3α, and sPLA2 were induced by carcinogen treatment, but repressed in rats fed either BB or SF. The expression for these genes was highest in the AIN(AOM-injected) group, and tended to parallel tumor incidence data. AOM-injected animals had a lower abundance of genes encoding somatostatin 2 receptor, carbonyl reductase, and amyloid beta (a4) precursor protein-binding, family b, member 3. The two former were generally higher in both BB and SF groups whereas the latter was elevated in the SF group only. Seven other transcripts occurred in both lists, however annotations were different, and when averaged, did not reach statistical significance for both diet and injection.

RT-PCR gene changes.

The abundance of five selected genes (CDC2, cyclin B1, TOP2A, GADD45α, and sPLA2) was confirmed by RT-PCR. These genes were chosen due to the known effects of cell proliferation and inflammation on tumorigenesis. As shown in **FIGURE 2,** the mRNA for CDC2, cyclin B1, and TOP2A were all significantly lower, whereas GADD45α was higher in the colon of BB and SF-fed rats compared to controls (AIN). The relative fold changes in sPLA2 are presented in **FIGURE 3**. In accordance with microarray data, there were significant main effects for both diet ($P < 0.009$) and carcinogen ($P < 0.006$) treatment. Generally, sPLA2 expression was lowest in bean-fed rats, but increased in all diets with carcinogen injections (fold-increases of 2.6,

TABLE 5. Genes significantly affected both by carcinogen (AOM) injection and dietary treatment in the distal colonic epithelium of male F344 rats.[1]

		SHAM			AOM		
Gene Symbol	**Gene Title**	**AIN**	**BB**	**SF**	**AIN**	**BB**	**SF**
Pla2g2a	phospholipase a2, group iia	1	0.6	0.3	2.5	1	1.2
--	NP defensin 3α	1	0.4	0.7	1.6	0.8	1
Col1a1	collagen, type 1, α1	1	0.4	0.7	1.6	0.9	1
Fn1	fibronectin 1	1	0.7	0.8	2	1.2	1
Sstr2	somatostatin receptor 2	1	1.4	1.7	0.7	1.1	1.2

[1] Data expressed as mean fold-change differences standardized to the AIN(saline-injected) group (n=4/group). There were significant main effects for injection type (saline *vs* AOM) and dietary treatment (AIN *vs* BB *vs* SF, $P<0.05$) for each gene listed.

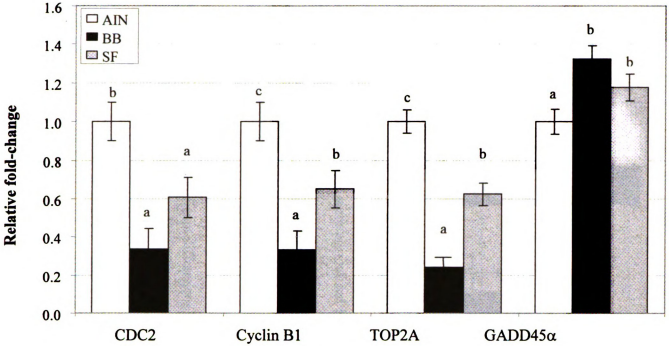


FIGURE 2. RT-PCR analysis of cell cycle-associated genes differentially affected by dietary treatment. Results were normalized to the housekeeping gene β-Actin and are presented as relative fold-changes (LSM ± SEM) standardized to the AIN(control) group. Bars with different superscripts denote significant main effects for diet for each gene presented ($P < 0.05$). *Abbreviations:* Cell division cycle 2 (CDC2), topoisomerase 2A (TOP2A), growth arrest and DNA damage induced 45α (GADD45α).

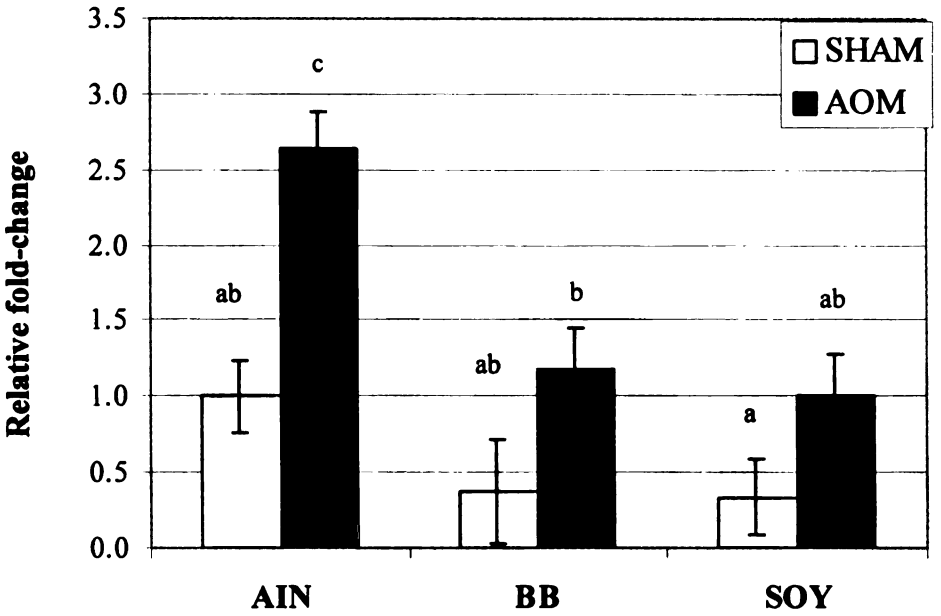


FIGURE 3. Relative fold-changes in sPLA2 mRNA abundance detected with RT-PCR. Results were normalized to the housekeeping gene β-Actin and are presented as mean fold-changes (LSM ± SEM) relative to the AIN(saline-injected) group. There were significant main effects for both diet ($P < 0.009$) and injection type ($P < 0.006$) on sPLA2 abundance. Superscripts denote significant differences between groups ($P < 0.05$).

3.1, and 3.1 for AIN, BB, and SF fed rats, respectively). Rats injected with AOM and fed the control (AIN) diet had the highest (2.6) overall expression level whereas mRNA expression in the BB(AOM-injected) (1.2) and SF(AOM-injected) (1.0) groups increased to values that were not different from the AIN(saline-injected) group (1.0).

E. DISCUSSION.

The focus of the current research was to ascertain potential cellular and molecular events underlying suppression of tumorigenesis by beans using a highly relevant animal model of colon cancer. We profiled global changes in gene expression affected by AOM-treatment in non-neoplastic colon mucosa to determine early events permissive for tumor formation and whether these changes could be attenuated by dietary treatment. Although not a primary focus of this study, tumor incidence was also assessed. As previously demonstrated, both BB (12) and SF-fed (8-11) rats developed significantly fewer tumors overall, confirming that these diets inhibit experimental colon carcinogenesis. In the study by Hangen and Bennink (12), however, black and navy bean fed-rats ate less, and as a result had significantly lower body weights at termination of the study. Because of the inverse association between energy restriction and tumorigenesis, the tryptophan and methionine content in the black bean diets was adjusted to raise the amino acid score comparable to that of the AIN and SF diets. As a result, no significant differences in final weight gain were detected, indicating that black beans inhibit tumorigenesis by a mechanism other than energy restriction.

Despite extensive use of the AOM model to study colon carcinogenesis, only a few studies have examined the molecular events associated with colon cancer

development. Davidson *et al.* (37) studied colon tissue 12 hours and 18 weeks after AOM injection, and suggested that AOM treatment caused changes in genes involved in apoptosis, although actual data were not presented. Other studies have examined molecular changes in microscopic lesions in the colon of carcinogen treated animals. For example, aberrant crypt foci (ACF) are lesions containing crypts that are larger in size, have altered lumenal openings, and exhibit thickened epithelia (38). ACF have been reported to occur in a dose-dependent manner following AOM-administration (38) and contain a high frequency of mutations in the proto-oncogene k-*ras* (29, 32). Activating mutations in β-catenin have also been reported in more dysplastic ACF, but nuclear accumulation of the protein is not frequently observed (39). Although ACF are considered to be putative precursors to adenomas, many ACF disappear and never develop into adenomas (40-41). On the other hand, Yamada and collegues (39, 42-45) recently described the presence of β-catenin accumulated crypts (BCAC) in *en face* preparations of rat colons following AOM-administration. BCAC are distinct from ACF, contain mutations in and nuclear accumulation of β-catenin, and exhibit disruptions in cellular morphology, dysplasia, and increased proliferative capacity (39, 42-45). Mutations affecting degradation of β-catenin or inactivation of the adenomatous polyposis coli (*APC*) gene are accepted to be initiating events in human (46-47) and rat colon tumorigenesis (31-32), respectively, thus BCAC may represent true premalignant lesions.

In this study, the global effects of AOM at 31 weeks post-treatment were associated with a higher expression of genes involved in innate defense and immunity. For example, the anti-microbial genes lysozyme and neutrophil (NP) defensin 3α were

approximately 2-fold higher than in saline injected controls. Lysozyme expression is characteristic of Paneth cell lineage, which are rarely found in the colon, but have been detected in dysplastic ACF (35), BCAC (42-44) and colon tumors (35). The presence of Paneth cells has been suggested to be a consequence of altered differentiation programming, possibly through aberrant activation of the Wnt/β-catenin pathway (43, 48). Several major histocompability MHC II-associated antigens were also induced in the colon of AOM-injected rats. Epithelial cells, activated dendritic cells, and/or macrophages underlying the intestinal cell layer can present MHC II antigens (49-52). A higher abundance of these transcripts implies enhanced immune responsiveness to lumenal antigens and suggests that AOM treatment alters mucosal barrier function. Soler (53) noted defects in tight-junction permeability in normal mucosa from carcinogen-treated animals, and the surface epithelial cells of ACF have also been reported to be deficient in mature goblet cells, have altered mucin composition, and contain irregular microvilli (54). A defective mucosal barrier that allows more antigenic materials to pass through the underlying lamina propria would be expected to result in low-grade inflammation and chronic inflammation has been implicated in the etiology of colon cancer (55-56). Although none of the well-known cell cycle-associated genes were altered by AOM-treatment, we detected moderate induction of ribosomal proteins, especially components of the 40S and 60S subunits. RNA and protein synthesis have been reported to decrease as cells terminally differentiate (57), and enhanced presence of ribosomal proteins has been noted in the colon of rat strains susceptible to PhIP-induced colon cancer (58) and in animals during aging (59).

The profile of genes altered by dietary treatment highlight differences in colon cell physiology with particular implications to carcinogenesis. For example, the most highly induced classes of genes affected by bean-diets included those involved in water channel and ion transport, oxidative electron transport, and fatty acid metabolism. These changes are consistent with the physiological effects of fermentable fibers in bean diets on colon epithelial function. Bacterial fermentation of dietary fiber and resistant starch produces the short-chain fatty acids (SCFA), acetate, propionate, and butyrate (60-61). SCFAs are known to be trophic to the colonic epithelium, enhance water and sodium absorption, increase mucosal blood flow, and modulate enterohormone release (reviewed in 62-64). Butyrate in particular has been demonstrated to inhibit cancer cell growth *in vitro* (65-66), and it has been suggested that butyrate metabolism, through mitochondrial β-oxidation, is important for induction of apoptosis *in vivo* (67). Downregulation of mitochondrial genes have been demonstrated in inflammatory conditions (36, 68), although it is not clear if this is a cause or effect relationship.

Alterations in crypt cellular kinetics associated with an expansion of the proliferative compartment towards the lumen has been described in non-neoplastic colon tissue from humans at risk for colon cancer development and in animals treated with carcinogens (69). In this study, several transcripts associated with proliferation and apoptosis were found to be regulated in a diet-dependent manner. An interesting finding was a 2-fold higher expression of CEACAM1 in rats fed either BB or SF. CEACAM1 is a cell-surface glycoprotein expressed in the differentiated cell compartment of colonic crypts that has been proposed to induce apoptotic signals in response to ankinois (70-71). Loss of CEACAM1 expression occurs with a similar frequency in hyperplastic polyps

and human colon cancers, and appears to precede defects in the *APC* pathway (71). Somatostatin receptor 2, which mediates anti-proliferative responses to the endocrine hormone somatostatin (72-73), was also more abundant in bean-fed animals and tended to decrease following AOM treatment. Whey and soy protein isolate have been reported to enhance both colonic mRNA and serum protein levels of somatostatin, suggesting neuroendocrine involvement in epithelial kinetics (74). Additionally, we detected lower mRNA abundance for the mitotic genes cyclin B1, CDC2, and TOP2A and higher GADD45α in bean-fed rats, indicative of a G2/M cell cycle arrest (75). TOP2A is involved in a variety of processes including DNA replication, chromosome segregation, and maintenance of chromosome structure (76). Protein expression has been detected primarily in the lower crypt compartment of human colons and levels increase during tumorigenesis (77). It is interesting that expression of these genes can be regulated in a p53-dependent manner (reviewed in 75), suggestive of enhanced capability for DNA repair and/or apoptotic responses in bean-fed animals. The anatomical distribution of TOP2A and CEACAM1 along the crypt-lumen axis concurrent with dietary modulation of other cell-cycle associated genes implies a general effect of bean diets on maintaining crypt cell homeostasis, potentially through enhancing cellular differentiation.

Of particular importance in this study with respect to dietary modulation of tumorigenesis were genes changes that paralleled tumor incidence data. Specifically, transcripts for sPLA2, NP defensin 3α, collagen 1α1, and fibronectin 1 were induced by AOM treatment in all diets, but less so in bean-fed animals. sPLA2 is induced in a variety of inflammatory and neoplastic conditions in the colon and other tissues (78-85). The protein exhibits anti-microbial activity (86), releases arachidonic acid from lipid

membranes (87-88), and has been demonstrated to activate neutrophils through catalytic mechanisms (arachidonic acid liberating) and independent of catalytic activity *in vitro* (89). Ikegami *et al.* (81) reported that in rats, colonic sPLA2 mRNA, but not cPLA2 or COX-2, increases acutely following carcinogen administration and is associated with higher levels of PGE2 and 6keto PGF1α. Production of prostaglandins would likely contribute to colon carcinogenesis through known effects on epithelial cell proliferation, apoptosis, and angiogenesis (90-93). Defensins are small cationic peptides, divided into α and β groups depending on the position of the inter-disulfide bonds between cysteine residues (94), and exhibit a range of antimicrobial acivity *in vitro* (95). NP defensin 3α was originally identified in neutrophil granules (96), however it has been detected in epithelial cells during severe inflammation (97) and in colon cancers (35). Similar to sPLA2, NP defensin 3α was induced by AOM injections in all diets, but levels were highest in AIN(casein)-fed animals. These results, in addition to a lower expression of toll-like receptor 4, a component of innate defense that initiates inflammation in response to bacterial lipopolysaccharide (98), implies that bean-feeding protects against AOM-induced mucosal barrier dysfunction.

The extracellular matrix (ECM) proteins fibronectin and collagen are involved in a variety of processes including proliferation, cell adhesion, and chemotaxis. Actively proliferating epithelial cells at the crypt base synthesize more fibronectin and levels decrease during differentiation (99). In addition, fibronectin has been identified as a downstream target of the Wnt/β-catenin pathway (100-101). Collagen 1α1 expression is upregulated in colon cancer (**CHAPTER III**) and in other hyperproliferative disorders (102), but normal colonic expression has not been reported. Dietary modulation of these

genes may represent a lower presence of BCAC and/or dysplastic cypts in bean-fed animals or a more general effect of diet on enhancing cellular differentiation.

In summary, this study provided insight into early molecular events associated with AOM-induced colon cancer as well as in chemoprevention by bean feeding. One of the major findings with respect to AOM-treatment was a higher expression of immune-related antigens, suggestive that dysfunctions in mucosa integrity are permissive for colon carcinogenesis. Bean-feeding induced changes in gene profiles consistent with modulation of crypt cytokinetics and energy metabolism. Specific molecular targets of beans that appear to corroborate reduced tumorigenesis include suppression of the genes sPLA2 and NP defensin 3α, as well as alterations in ECM components, which may affect migration and/or differentiation programming. We speculate that these gene changes coincide with the ability of bean diets to protect against mucosal barrier dysfunction, thereby suppressing microbial-induced inflammation. Future studies examining this possibility in more detail are warranted.

F. LITERATURE CITED.

1. American Cancer Society. (2005) Cancer Facts & Figures 2005. http://www.cancer.org/downloads/STT/CAFF2005f4PWSecured.pdf.

2. American Institute of Cancer Research/World Cancer Research Fund (AICR/WCRF). (1997) Colon, rectum. In *Food, Nutrition and the Prevention of Cancer: A Global Perspective,* BANTA Book Group, Menasha, WI, pp. 216-51.

3. Potter JD. (1999) Colorectal cancer: Molecules and populations. *Journal of the National Cancer Institute.* 91(11): 916-932.

4. Steinmetz KA and Potter JD. (1993) Food-group consumption and colon cancer in the Adelaide Case-Control Study. I. Vegetables and fruit. *Int J Cancer.* 53(5): 711-9.

5. Singh P N and Fraser GE. (1998) Dietary risk factors for colon cancer in a low-risk population. *American Journal of Epidemiology.* 148: 761-774

6. Fraser GE. (1999) Associations between diet and cancer, ischemic heart disease, and all-cause mortality in non-Hispanic white California Seventh-day Adventists. *American Journal of Clinical Nutrition.* 70: 532S-538S

7. Correa P. (1981) Epidemiological correlations between diet and cancer frequency. *Cancer Research.* 41: 3685-3689

8. Bennink MR and Om AS. (1998) Inhibition of colon cancer (CC) by soy phytochemicals but not by soy protein. *Faseb Journal.* 12(5): 3808.

9. Bennink MR, Om AS and Miyagi Y. (1999) Inhibition of colon cancer (CC) by soy flour but not by genistin or a mixture of isoflavones. *Faseb Journal.* 13(4): A50-A50.

10. Bennink MR, Om AS and Miyagi Y. (2000) Inhibition of colon cancer (CC) by wheat bran, soy flour, and flax. *Faseb Journal.* 14(4): A217-A217.

11. Rondini EA and Bennink MR. (2002) Defatted soy flour, but not soy concentrate, reduces azoxymethane-induced colon carcinogenesis. *Journal of Nutrition.* 132(3): 589S-589S.

12. Hangen LA and Bennink MR. (2003) Consumption of black beans and navy beans (Phaseolus vulgaris) reduced axozymethane-induced colon cancer in rats. *Nutrition and Cancer.* 44: *60-6*

13. Hughes JS, Ganthavorn C, and Wilson-Sanders S. (1997) Dry beans inhibit azoxymethane-induced colon carcinogenesis in F344 rats. *Journal of Nutrition.* 127: 2328-2333.

14. Hakkak R, Korourian S, Ronis MJJ *et al.* (2001) Soy protein isolate consumption protects against azoxymethane-induced colon tumors in male rats. *Cancer Letters.* 166(1): 27-32.

15. Raicht RF, Cohen BI, Fazzini EP *et al.* (1980) Protective effect of plant sterols against chemically induced colon tumors in rats. *Cancer Res.* 40(2): 403-5.

16. Weed HG, McGandy RB and Kennedy AR. (1985) Protection against Dimethylhydrazine-Induced Adenomatous Tumors of the Mouse Colon by the Dietary Addition of an Extract of Soybeans Containing the Bowman-Birk Protease Inhibitor. *Carcinogenesis.* 6(8): 1239-1241.

17. Shamsuddin AM, Elsayed AM and Ullah A. (1988) Suppression of Large Intestinal Cancer in F344 Rats by Inositol Hexaphosphate. *Carcinogenesis.* 9(4): 577-580.

18. Shamsuddin AM and Ullah A. (1989) Inositol Hexaphosphate Inhibits Large Intestinal Cancer in F344 Rats 5 Months after Induction by Azoxymethane. *Carcinogenesis.* 10(3): 625-626.

19. Ullah A and Shamsuddin AM. (1990) Dose-Dependent Inhibition of Large Intestinal Cancer by Inositol Hexaphosphate in F344 Rats. *Carcinogenesis.* 11(12): 2219-2222.

20. Billings PC, Newberne PM and Kennedy AR. (1990) Protease Inhibitor Suppression of Colon and Anal Gland Carcinogenesis Induced by Dimethylhydrazine. *Carcinogenesis.* 11(7): 1083-1086.

21. Koratkar R and Rao AV. (1997) Effect of soya bean saponins on azoxymethane-induced preneoplastic lesions in the colon of mice. *Nutrition and Cancer-an International Journal.* 27(2): 206-209.

22. Fotsis T, Pepper MS, Aktas E *et al.* (1997) Flavonoids, dietary-derived inhibitors of cell proliferation and *in vitro* angiogenesis. *Cancer Res.* 57: 2916-2921.

23. Wenzel H, Kuntz S, Brendel MD and Daniel H. (2000) Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells. *Cancer Research.* 60(14): 3823-3831.

24. Nguyen DT, Garcia J and Cadenas E. (2001) Genistein causes cell cycle arrest and apoptosis via inhibition of p53-independent mechanisms in T47D breast cancer cells. *Free Radical Biology and Medicine.* 31: (S105-S105).

25. Sandra F, Matsuda M, Yoshida H and Hirata M. (2002) Inositol hexakisphosphate blocks tumor cell growth by activating apoptotic machinery as well as by inhibiting the Akt/NF kappa B-mediated cell survival pathway. *Carcinogenesis.* 23(12): 2031-2041.

26. Kennedy AR, Billings PC, Wan XS and Newberne PM. (2002) Effects of Bowman-Birk inhibitor on rat colon carcinogenesis. *Nutrition and Cancer-an International Journal.* 43(2): 174-186.

27. Yu ZL, Li WJ and Liu FY. (2004) Inhibition of proliferation and induction of apoptosis by genistein in colon cancer HT-29 cells. *Cancer Letters.* 215(2): 159-166.

28. Jacoby RF, Llor X, Teng BB *et al.* (1991) Mutations in the K-Ras Oncogene Induced by 1,2-Dimethylhydrazine in Preneoplastic and Neoplastic Rat Colonic Mucosa. *Journal of Clinical Investigation.* 87(2): 624-630.

29. Vivona AA, Shpitz B, Medline A *et al.* (1993) K-Ras Mutations in Aberrant Crypt Foci, Adenomas and Adenocarcinomas During Azoxymethane-Induced Colon Carcinogenesis. *Carcinogenesis.* 14(9): 1777-1781.

30. Pories SE, Ramchurren N, Summerhayes I and Steele G. (1993) Animal models for colon carcinogenesis. *Archives of Surgery.* 128: 647-53.

31. Takahashi M, Fukuda K, Sugimura T and Wakabayashi K. (1998) Beta-catenin is frequently mutated and demonstrates altered cellular location in azoxymethane-induced rat colon tumors. *Cancer Research.* 58(1): 42-46. ACF

32. Takahashi M and Wakabayashi K. (2004) Gene mutations and altered gene expression in azoxymethane-induced colon carcinogenesis in rodents. *Cancer Science.* 95(6): 475-480.

33. Liu T, Mokuolo AQ, Rao CV *et al.* (1995) Regional chemoprevention of carcinogen-induced tumors in rat colon. *Gastroenterology.* 109: 1167-72.

34. Reeves PG, Nielsen FH and Fahey GC. (1993) Ain-93 Purified Diets for Laboratory Rodents - Final Report of the American Institute of Nutrition Ad Hoc Writing Committee on the Reformulation of the Ain-76a Rodent Diet. *Journal of Nutrition.* 123(11): 1939-1951.

35. Fujiwara K, Ochiai M, Ohta T *et al.* (2004) Global gene expression analysis of rat colon cancers induced by a food-borne carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. *Carcinogenesis.* 25(8): 1495-1505.

36. Lawrance IC, Fiocchi C and Chakravarti S. (2001) Ulcerative colitis and Crohn's disease: distinctive gene expression profiles and novel susceptibility candidate genes. *Human Molecular Genetics.* 10(5): 445-56.

37. Davidson LA, Nguyen DV, Hokanson RM *et al.* (2004) Chemopreventive n-3 polyunsaturated fatty acids reprogram genetic signatures during colon cancer initiation and progression in the rat. *Cancer Research.* 64(18): 6797-6804.

38. Bird RP. (2005) Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. *Cancer Lett.* 93: 55-71.

39. Yamada Y, Yoshimi N, Hirose Y *et al.* (2000) Frequent beta-catenin gene mutations and accumulations of the protein in the putative preneoplastic lesions lacking macroscopic aberrant crypt foci appearance, in rat colon carcinogenesis. *Cancer Research.* 60(13): 3323-3327.

40. Hardman WE, Cameron IL, Heitman DW and Contreras E. (1991) Demonstration of the Need for End-Point Validation of Putative Biomarkers - Failure of Aberrant Crypt Foci to Predict Colon Cancer Incidence. *Cancer Research.* 51(23): 6388-6392.

41. Davies MJ, Bowey EA, Adlercreutz H *et al.* (1999) Effects of soy or rye supplementation of high-fat diets on colon tumour development in azoxymethane-treated rats. *Carcinogenesis.* 20(6): 927-931.

42. Yamada Y, Yoshimi N, Hirose Y *et al.* (2001) Sequential analysis of morphological and biological properties of beta-catenin-accumulated crypts, provable premalignant lesions independent of aberrant crypt foci in rat colon carcinogenesis. *Cancer Research.* 61(5): 1874-1878.

43. Yamada Y and Mori H. (2003) Pre-cancerous lesions for colorectal cancers in rodents: a new concept. *Carcinogenesis.* 24(6): 1015-1019.

44. Hirose Y, Kuno T, Yamada Y *et al.* (2003) Azoxymethane-induced beta-catenin-accumulated crypts in colonic mucosa of rodents as an intermediate biomarker for colon carcinogenesis. *Carcinogenesis.* 24(1): 107-111.

45. Mori H, Yamada Y, Kuno T and Hirose Y. (2004) Aberrant crypt foci and beta-catenin accumulated crypts; significance and roles for colorectal carcinogenesis. *Mutation Research-Reviews in Mutation Research;* 566(3): 191-208.

46. Fearon ER and Vogelstein B. (1990) A Genetic Model for Colorectal Tumorigenesis. *Cell.* 61(5): 759-767.

47. Kinzler KW and Vogelstein B. (1996) Lessons from hereditary colorectal cancer. *Cell.* 87(2): 159-170.

48. Andreu P, Colnot S, Godard C *et al.* (2005) Crypt-restricted proliferation and commitment to the Paneth cell lineage following Apc loss in the mouse intestine. *Development.* 132(6): 1443-1451.

49. Nagashima R, Maeda K, Imai Y and Takahashi T. (1996) Lamina propria macrophages in the human gastrointestinal mucosa: Their distribution, immunohistological phenotype, and function. *Journal of Histochemistry & Cytochemistry.* 44(7): 721-731.

50. Maric I, Holt PG, Perdue MH and Bienenstock J. (1996) Class II MHC antigen (Ia)-bearing dendritic cells in the epithelium of the rat intestine. *Journal of Immunology.* 156(4): 1408-1414.

51. German AJ, Bland PW, Hall EJ and Day MJ. (1998) Expression of major histocompatibility complex class II antigens in the canine intestine. *Veterinary Immunology and Immunopathology.* 61(2-4): 171-180.

52. Macdonald TT. (2003) The mucosal immune system. *Parasite Immunology;* 25(5): 235-246.

53. Soler AP, Miller RD, Laughlin KV *et al.* (1999) Increased tight junctional permeability is associated with the development of colon cancer. *Carcinogenesis.* 20(8): 1425-1431.

54. Vaccina F, Scorcioni F, Pedroni M *et al.* (1998) Scanning electron microscopy of aberrant crypt foci in human colorectal mucosa. *Anticancer Research.* 18(5A): 3451-3456.

55. O'Byrne KJ and Dalgleish AG. (2001) Chronic immune activation and inflammation as the cause of malignancy. *Br J Cancer.* 85(4): 473-83.

56. Huls G, Koornstra JJ and Kleibeuker JH. (2003) Non-steroidal anti-inflammatory drugs and molecular carcinogenesis of colorectal carcinomas. *Lancet.* 362(9379): 230-232.

57. Deschner EE and Lipkin M. (1970) Study of Human Rectal Epithelial Cells in-Vitro .3. Rna Protein and DNA Synthesis in Polyps and Adjacent Mucosa. *Journal of the National Cancer Institute.* 44(1): 175-.

58. Fujiwara K, Ochiai M, Ubagai T *et al.* (2003) Differential gene expression profiles in colon epithelium of two rat strains with distinct susceptibility to colon carcinogenesis after exposure to PhIP in combination with dietary high fat. *Cancer Science.* 94(8): 672-678.

59. Lee HM, Greeley GH and Englander EW. (2001) Age-associated changes in gene expression patterns in the duodenum and colon of rats. *Mechanisms of Ageing and Development;* 122(4): 355-371.

60. Cummings JH. (1981) Short chain fatty acids in the human colon. *Gut.* 22(9): 763-79.

61. Cummings JH. and Macfarlane, G.T. (1991) The control and consequences of bacterial fermentation in the human colon. *J. Appl. Bacter.* 70: 443-59.

62. Cook SI and Sellin JH. (1998) Review article: short chain fatty acids in health and disease. *Alimentary Pharmacology & Therapeutics.* 12(6): 499-507.

63. Blottiere HM, Champ M, Hoebler C, Michel C and Cherbut C. (1999) Production and digestive effects of short chain fatty acids. *Sciences Des Aliments.* 19(3-4): 269-290.

64. Miller SJ. (2004) Cellular and physiological effects of short-chain fatty acids. *Mini-Reviews in Medicinal Chemistry.* 4(8): 839-845.

65. Hague A, Manning AM, Huschtscha LI *et al.* (1993) Sodium butyrate induces apoptosis in human colonic tumor cell lines in a p-53 independent pathway: implications for the possible role of dietary fiber in the prevention of large bowel cancer. *Int. J. Cancer.* 55: 498-505.

66. Heerdt BG, Houston MA, and Augenlicht LH. (1997) Short-chain fatty acid-initiated cell cycle arrest and apoptosis of colonic epithelial cells is linked to mitochondrial function. *Cell Growth & Differentiation.* 8: 523-32.

67. Augenlicht LH, Anthony GM, Church TL *et al.* (1999) Short-chain fatty acid metabolism, apoptosis, and Apc-initiated tumorigenesis in the mouse gastrointestinal mucosa. *Cancer Research.* 59(23): 6005-6009.

68. Birkenkamp-Demtroder K, Christensen LL, Olesen SH *et. al.* (2002) Gene expression profiling in colorectal cancer. *Cancer Research.* 62: 4352-4363.

69. Lipkin M. (1974) Phase 1 and Phase 2 Proliferative Lesions of Colonic Epithelial-Cells in Diseases Leading to Colonic Cancer. *Cancer.* 34(3): 878-888.

70. Shively JE. (2004) CEACAM1 and hyperplastic polyps: new links in the chain of events leading to colon cancer. *Oncogene.* 23(58): 9303-9305.

71. Nittka S, Gunther J, Ebisch C, Erbersdobler A and Neumaier M. (2004) The human tumor suppressor CEACAM1 modulates apoptosis and is implicated in early colorectal tumorigenesis. *Oncogene.* 23(58): 9306-9313.

72. Buscail L, Esteve JP, Saintlaurent N *et al.* (1995) The Somatostatin Receptor Subtype-Sstr2 and Subtype-Sstr5 Mediate the Inhibition of Cell-Proliferation. *Gastroenterology.* 108(4): A955-A955.

73. Vernejoul F, Faure P, Benali N *et al.* (2002) Antitumor effect of in vivo somatostatin receptor subtype 2 gene transfer in primary and metastatic pancreatic cancer models. *Cancer Research.* 62(21): 6124-6131.

74. Xiao R, Badger TM and Simmen FA. (2004) Dietary exposure to soy or whey proteins alters colonic global gene expression profiles during rat colon tumorigenesis. *Mol Cancer.* 11: 4(1).

75. Taylor WR and Stark GR. (2001) Regulation of the G2/M transition by p53. *Oncogene.* 20(15): 1803-1815.

76. Bakshi RP, Galande S and Muniyappa K. (2001) Functional and regulatory characteristics of eukaryotic type II DNA topoisomerase. *Critical Reviews in Biochemistry and Molecular Biology.* 36(1): 1-37.

77. Boman BM, Walters R, Fields JZ *et al.* (2004) Colonic crypt changes during adenoma development in familial adenomatous polyposis - Immunohistochemical evidence for expansion of the crypt base cell population. *American Journal of Patholog.* 165(5): 1489-1498.

78. Nevalainen TJ. (1993) Serum Phospholipases A(2) in Inflammatory Diseases. *Clinical Chemistry.* 39(12): 2453-2459.

79. Minami T, Tojo H, Shinomura Y *et al.* (1994) Increased Group-Ii Phospholipase a(2) in Colonic Mucosa of Patients with Crohns-Disease and Ulcerative-Colitis. *Gut.* 35(11): 1593-1598.

80. Yamashita S, Ogawa M, Sakamoto K *et al.* (1994) Elevation of Serum Group-Ii Phospholipase a(2) Levels in Patients with Advanced Cancer. *Clinica Chimica Acta.* 228(2): 91-99.

81. Ikegami T, Matsuzaki Y, Shoda J *et al.* (1998) The chemopreventive role of ursodeoxycholic acid in azoxymethane-treated rats: suppressive effects on enhanced group II phospholipase A(2) expression in colonic tissue. *Cancer Letters.* 134(2): 129-139.

82. Haapamaki MM, Gronroos JM, Nurmi H *et al.* (1997) Gene expression of group II phospholipase A2 in intestine in ulcerative colitis. *Gut.* 40(1): 95-101.

83. Ogawa M. (1997) Group II phospholipase A(2) in neoplastic diseases. *Phospholipase A2.* 24: 200-204.

84. Haapamaki MM, Gronroos JM, Nurmi H *et al.* (1999) Phospholipase A(2) in serum and colonic mucosa in ulcerative colitis. *Scandinavian Journal of Clinical & Laboratory Investigation.* 59(4): 279-287.

85. Nevalainen TJ, Haapamaki MM and Gronroos JM. (2000) Roles of secretory phospholipases A(2) in inflammatory diseases and trauma. *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids.* 1488(1-2): 83-90.

86. Buckland AG and Wilton DC. (2000) The antibacterial properties of secreted phospholipases A(2). *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids.* 1488(1-2): 71-82.

87. Bingham CO and Austen KF. (1999) Phospholipase A(2) enzymes in eicosanoid generation. *Proceedings of the Association of American Physicians.* 111(6): 516-524.

88. Marshall LA and Morgan DW. (1998) Modulation of arachidonic acid metabolism: Focus on phospholipase A(2)(s). *Drug News & Perspectives.* 11(2): 82-91.

89. Zallen G, Moore EE, Johnson JL *et al.* (1998) New mechanisms by which secretory phospholipase A(2) stimulates neutrophils to provoke the release of cytotoxic agents. *Archives of Surgery.* 133(11): 1229-1232.

90. Herschman HR. (1998) Recent progress in the cellular and molecular biology of prostaglandin synthesis. *Trends in Cardiovascular Medicine.* 8(4): 145-150.

91. Ashktorab H, Dawkins FW, Mohamed R *et al.* (2005) Apoptosis induced by aspirin and 5-fluorouracil in human colonic adenocarcinoma cells. *Digestive Diseases and Sciences.* 50(6): 1025-1032.

92. Tarnawski AS and Jones MK. (2003) Inhibition of angiogenesis by NSAIDs: molecular mechanisms and clinical implications. *Journal of Molecular Medicine-Jmm.* 81(10): 627-636.

93. Williams CS, Mann M and DuBois RN. (1999) The role of cyclooxygenases in inflammation, cancer, and development. *Oncogene.* 18(55): 7908-7916.

94. Cunliffe RN. (2003) alpha-Defensins in the gastrointestinal tract. *Molecular Immunology.* 40(7): 463-467.

95. Ganz T and Lehrer RI. (1994) Defensins. *Current Opinion in Immunology..*6(4): 584-589.

96. Eisenhauer PB, Harwig SSL, Szklarek D *et al.* (1989) Purification and antimicrobial properties of three defensins from rat neutrophils. *Infect. Immun.* 57: 2021-2027.

97. Cunliffe RN, Kamal M, Rose F *et al.* (2002) Expression of antimicrobial neutrophil defensins in epithelial cells of active inflammatory bowel disease mucosa. *Journal of Clinical Pathology.* 55(4): 298-304.

98. Ohkawara T, Takeda, H, Miyashita K *et al.* (2005) Regulation of toll-like receptor 4 expression in mouse colon by macrophage migration inhibitory factor. *Histochem. Cell Biol.* 9: 1-8.

99. Vachon PH, Simoneau A, Herringgillam FE and Beaulieu JF. (1995) Cellular Fibronectin Expression Is Down-Regulated at the Messenger-Rna Level in Differentiating Human Intestinal Epithelial-Cells. *Experimental Cell Research.* 216(1): 30-34.

100. Gradl D, Kuhl M and Wedlich D. (1999) The Wnt/Wg signal transducer beta-catenin controls fibronectin expression. *Molecular and Cellular Biology.* 19(8): 5576-5587.

101. Takayasu H, Horie H, Hiyama E *et al.* (2001) Frequent deletions and mutations of the beta-catenin gene are associated with overexpression of cyclin D1 and fibronectin and poorly differentiated histology in childhood hepatoblastoma. *Clinical Cancer Research.* 7(4): 901-908.

102. deGiorgio-Miller AM, Treharne LJ, McAnulty RJ *et al.* (2005) Procoltagen type I gene expression and cell proliferation are increased in lipodermatoscterosis. *British Journal of Dermatology.* 152(2): 242-249.

CHAPTER V.

BLACK BEANS AND SOY FLOUR REDUCE INFLAMMATORY BIOMARKERS AND RESTORE COLONOCYTE DIFFERENTIATION DURING AOM-INDUCED COLON CARCINOGENESIS IN RATS

A. ABSTRACT.

We have recently demonstrated that that dietary suppression of carcinogen-induced colon cancer by beans was associated with modulation of mucosal genes involved in cellular kinetics, energy metabolism, and innate immunity in rats. Additionally, we identified sPLA2 as a potential modifier gene in this model, being induced by AOM administration and suppressed by bean-feeding. sPLA2 is involved in a variety of processes including anti-microbial defenses and inflammation. Therefore, this study was conducted to further explore the involvement of this protein in colon cancer by addressing whether induction of sPLA2 was associated with alterations in epithelial cellular kinetics and immune responsiveness during the early stages of AOM-induced colon carcinogenesis. Male F344 rats were obtained at 3 weeks of age and placed on a control (AIN) diet. At 4 and 5 weeks of age, animals were administered subcutaneous injections of AOM (n=60, 13 mg/kg) or saline (n=20). Beginning at 6 weeks of age, rats injected with AOM were separated into one of three dietary treatments (AIN, black beans (BB), or soy flour (SF)). Animals were sacrificed at 21 weeks of age and a section of the medial half of the distal colon was processed to assess changes in epithelial proliferation, sPLA2, and macrophage infiltration by immunohistochemistry. Rats injected with AOM and fed the control diet (AIN) exhibited a higher percentage of proliferative cells in the middle crypt compartment compared to rats fed BB, SF, or control-fed animals not administered carcinogen. A positive correlation was found between sPLA2 immunoreactivity and the zone of proliferative cells. Macrophage infiltration was also modestly higher in animals fed the control diet and administered AOM, suggesting a generalized effect of bean diets on reducing colonic inflammation. We concluded that

early induction in sPLA2 is related to hyperplastic changes in colonic mucosa. The ability of beans to suppress colon cancer is associated with modulating early biomarkers of inflammation, potentially through enhancing cellular differentiation.

B. INTRODUCTION.

Dietary habits are strongly associated with the incidence of and mortality from colorectal cancer (CRC) (1,2). Several experimental studies conducted in animals have demonstrated that both dry beans and soy flour inhibit carcinogen-induced colon cancer (3-8). Using microarrays, we recently reported that dietary suppression of colon cancer in rats was associated with modulation of mucosal genes involved in cellular kinetics, energy metabolism, and innate immunity **(CHAPTER IV)**. More specifically, we identified group IIA secretory phospholipase A2 (sPLA2), a pro-inflammatory protein, as a potential target for CRC inhibition by beans. sPLA2 is involved in a variety of processes, including anti-microbial defenses (9), sustained arachidonic acid release from membranes (10), and activation of macrophages and neutrophils (11-13). The protein is constitutively expressed in gland cells of some tissues including lacrimal, prostatic, and seminal glands and in Paneth cells of the small intestine, suggesting anti-microbial activity is important for host defenses (14). However, induction of sPLA2 in several disease states including multi system organ failure, cardiovascular disease, ulcerative colitis, and cancer indicates sPLA2 may perpetuate inflammation, likely through catalytic production of lipid mediators (14-20).

In humans and in animals, sPLA2 is normally expressed at low levels in the colon. Kennedy *et al.* (21) reported elevated levels of mRNA for sPLA2 in tumors from humans with familial adenomatous polyposis (FAP), who have inherited susceptibility to

colon cancer development. Edhemovic *et al.* (22) also demonstrated a stronger immunoreactivity of sPLA2 in the peritumoral mucosa than sites more distal to or within the colon tissue. Additionally, in animals, administration of carcinogens to initiate colon cancer enhances sPLA2 mRNA and protein expression (23-24). These results suggest a role for sPLA2 in tumorigenesis, although the mechanisms involved have not been elucidated. Igemagi *et al.* (23) found a correlation between sPLA2 activity and production of the prostaglandins PGE_2 and 6-keto PGF_1 in colonic mucosa of animals treated with AOM. PGE_2 has been associated with promotion of colon cancer by stimulating epithelial proliferation and inhibiting apoptosis (25-27). Additionally, tumor tissue is heavily infiltrated with immune cells, and *in vitro*, sPLA2 stimulates superoxide and nitric oxide release from neutrophils and in macrophages primed with the bacterial antigen lipopolysaccharide (LPS) (11-13). Thus, inability to suppress sPLA2 may promote tumorigenesis indirectly by stimulating inflammatory cells to release by-products, which can result in epithelial damage. The focus of the current investigation was to determine if AOM-induced changes in sPLA2 expression were related to alterations in colonic epithelial kinetics in rats and whether these changes could be attenuated by bean-feeding. Macrophage infiltration in colonic tissue was also measured to assess generalized dietary differences in inflammation.

C. MATERIALS AND METHODS.

Animal care and experimental diets.

This study was conducted in accordance with procedural guidelines of the Michigan State University Committee on Animal Use and Care. Three-week old male

Fischer (F344) rats (n=90) were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and housed in plastic cages (2-3 rats/cage) in temperature (23°C ± 2°) and humidity (40-60%) controlled rooms with a 12 hour light/dark cycle. Animals were observed daily for health status and body weights were monitored on a weekly basis. Throughout the experiment, animals had free access to food and house distilled water.

The experimental diets utilized in this study have previously been described in detail **(CHAPTER IV)**. Briefly, three diets were formulated based on the AIN-93G rodent diet and contained 1) casein (AIN), 2) cooked, dried, ground black beans (BB), or 3) defatted soy flour (SF; Archer Daniels Midland; Decatur, IL) as the protein source. All diets contained approximately 18.9% (wt/wt) total protein, 11.3% fiber, and 16.7% fat (lard: soybean oil mixture).

Experimental procedures.

Animals were fed the control (AIN) diet upon arrival, and after a one week acclimatization period, were injected once per week for two weeks with 13 mg/kg of azoxymethane (AOM) (Sigma Chemical Co., St. Louis, MO) prepared in saline (n=60) or saline (SHAMs, n=20). One week following injections (7 weeks of age), animals treated with AOM were randomly assigned to either continue on the AIN (control) diet or receive experimental diets (BB, SF). Saline-injected animals were fed the control (AIN) diet throughout the course of the experiment. At 21 weeks of age, animals were sacrificed by affixation with carbon dioxide and exsanguination, and colons were immediately excised, opened longitudinally, and rinsed in tap water to remove debris. The colon was transected into proximal and distal segments, and a section (2 cm) from the medial half of the distal colon was excised, pinned flat on cardboard and fixed in 10%

neutral buffered formalin (NBF, pH 7.4) for 4-6 hours. Tissues were then dehydraded in graded ethanol-water baths, rinsed in xylene, and infiltrated with paraffin prior to immunohistochemical procedures (see below).

Immunohistochemistry.

Dietary influences on colonic expression of PCNA, sPLA2, and CD68+ macrophages were examined by peroxidase biotin-streptavidin immunohistochemistry. Anti-human monoclonal antibody against group IIA PLA2 (sPLA2) was purchased from Caymen Chemical Co. (Ann Arbor, MI) and anti-rat CD68 monoclonal antibody from Ab-CAM, Inc. (Cambridge, MA). Anti-human monoclonal antibody against proliferating cell nuclear antigen (PCNA) and secondary antibodies were purchased from DAKO (Carpinteria, CA). The remaining reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Four-micron thick sections of colon tissue were placed on poly-*L*-lysine coated slides and affixed to the slides by drying at 58 °C for 2 hours, deparaffinized in xylene, and rehydrated in ethanol-H_2O with a final rinse in H_2O. For antigen retrieval, slides were immersed in a 10 mM citrate buffer (pH 6.0) containing 0.05% Tween-20 and placed in a steamer for 30 minutes at 92-95 °C. After cooling to room temperature (20 minutes), sections were treated with a 3% H_2O_2 solution for 10 minutes to block endogenous peroxidase activity, followed by a 1% bovine serum albumin (BSA) solution prepared in Tris-buffered saline (TBS; 10 mM tris(hydroxymethyl)aminomethane-buffered saline, pH 7.4) for 30 minutes to reduce non-specific binding of antibody. Sections were then incubated overnight at 4 °C with primary antibodies diluted 1:500 in TBS buffer containing 1% BSA. After extensive washing, tissue sections were treated

with biotinylated rabbit anti-mouse immunoglobulins followed by peroxidase-conjugated streptavidin at room temperature for 1 hour each. Antigen-linked peroxidase was detected with the chromagen 3-3'-diaminobenzidine (DAB) diluted in a 10 mM phosphate buffered saline solution (PBS, pH 7.2) containing 0.015% H_2O_2 for 5 minutes. Tissues were then lightly stained in Gill's hemotoxylin, blued in 0.3% ammonium water, then dehydrated in ethanol-xylene baths before mounting with Permount. All antibody incubations were performed in Shandon racks and sections were rinsed with 10 mM TBS (pH 7.4) between steps.

Quantitative analysis of immunohistochemistry.

For quantification of PCNA labeled cells, a researcher blinded to treatments evaluated 10-20 full-length crypts/animal. The total number of nuclei ($PCNA^+$ and $PCNA^-$) lining one side of the crypt and extending from the base of the crypt (cell 1) to the lumen was recorded. Crypt height (average number cells/hemicrypt), labeling index (number of $PCNA^+$ cells/crypt height) and proliferation zone (highest labeled $PCNA^+$ cell/crypt height) were then calculated and analyzed. Colonic staining of sPLA2 and CD68+ were quantified using BioQuant Imaging Software (Nashville, TN) and a light microscope (Nikon Optiphot-2; Kanagawa, Japan) equipped with a color video camera (Sony, Hyper HAD; New York, NY). For sPLA2 determination a 40X objective was used. Individual crypts projected on the computer screen were traced with a cursor, and the thresholded stain was measured. Due to the high degree of background with sPLA2 staining, only the deeper part of the stain near the basolateral surface was quantified. The heights of each crypt, extending from the base to the lumen, were also measured and output values calibrated with a micrometer. CD68+ macrophages were quantitated using

a 20X objective. The lamina propria between and below crypts was traced and the positive stained area (total number of pixels) were quantified. Data are expressed as a percentage of positive stained area (macrophages) in relation to the total surface area. For each stain, 10 measurements/animal were taken.

Statistical analyses.

Statistical analyses for body weights and immunohistochemical data were performed using the General Linear Model procedure of SAS (SAS Institute, Inc. Cary, NC, Version 7.0) and when appropriate, least squares means (LSM) were compared using the least significant difference method. Correlation and linear regression analysis were performed using Microsoft Excel 2000.

D. RESULTS.

Body weights.

There was no significant effect on body weight gain between groups. The final weight gain (g, LSM ± SEM) per group was: AIN(SHAM) = 214 ± 4.2, AIN(AOM)= 222 ± 4.1, BB(AOM) = 216 ± 4.1, SF(AOM) = 209 ± 4.1.

PCNA and sPLA2 immunohistochemistry.

Results for PCNA proliferation and sPLA2 indices are presented in **TABLE 1**. There was no effect of treatment on whole crypt labeling index (P = 0.07). However, significant differences were detected between groups in the percent of labeled cells per crypt compartment and in the proliferation zone (highest PCNA+ cell/crypt). Rats injected with AOM and fed the AIN diet had a higher proliferation zone (P = 0.0005) compared to rats fed BB or SF or the control (saline-injected) group. Similar treatment effects were also evident for the percent of proliferative cells in the middle crypt

compartment and sPLA2 immunoreactivity **(TABLE 1,** P = 0.0001). sPLA2 was localized primarily in the lower crypt compartment towards the basolateral surface **(FIGURE 1).** Further, a highly significant correlation between sPLA2 and the proliferation zone (r = 0.86, P = 0.0001) was detected indicating 74% of the differences in PZ between groups were related, in this study, to differences in the amount of sPLA2 **(FIGURE 2).**

Presence of CD68+ macrophages in colonic mucosa.

There was a modest, although significant effect of diet on macrophage infiltration into the lamina propria (P = 0.04). Consistent with sPLA2 data, rats fed the control diet and injected with AOM had a higher presence of macrophages than either rats fed the BB or SF diets or in control-fed, saline-injected animals. Group means were 0.68%, 0.81%, 0.62%, and 0.64% for AIN(saline), AIN(AOM), BB(AOM), and SF(AOM) groups, respectively **(FIGURE 3).** Tumor tissue from a separate study was included as a positive control (5.4%). As shown in **FIGURE 4,** a majority of macrophages were situated immediately below the epithelial layer near the crypt lumen.

TABLE 1. Main effects of treatment on PCNA proliferation indices and sPLA2 intensity in the distal colonic epithelium of male F44 rats.[1]

	PCNA labeling		PCNA labeling index per crypt compartment			sPLA2 intensity
Group	Labeling Index	Proliferative Zone	Bottom 1/3	Middle 1/3	Top 1/3	sPLA2 intensity/ crypt height [2]
AIN 93G (Saline-injected)	0.51	0.56 [a]	0.97	0.56 [a]	0.002 [a]	1.4 ± 0.13 [a]
AIN 93G (AOM-injected)	0.54	0.60 [b]	0.96	0.63 [b]	0.008 [b]	2.2 ± 0.14 [b]
Black Bean (AOM-injected)	0.51	0.56 [a]	0.97	0.55 [a]	0 [a]	1.0 ± 0.13 [a]
Soy Flour (AOM-injected)	0.51	0.55 [a]	0.96	0.56 [a]	0.002 [a]	1.2 ± 0.12 [a]
P Value	0.07	0.0005	0.69	0.04	0.001	0.0001

1 Data presented as LSM ± SEM based on a sample size of 8-10 rats/group. Superscripts denote significant differences in group means per column.

2 sPLA2 intensities were quantified using BioQuant imaging Software. Intensity is defined as the average number of thresholded pixels (stain) within a crypt. Crypt height was calibrated and expressed in micron units.

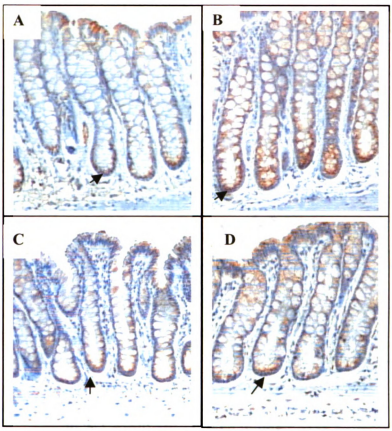


FIGURE 1. Immunoreactivity for sPLA2 in colonic mucosa. (A) AIN-saline-injected, (B) AIN(AOM-injected), (C) BB(AOM-injected), and (D) SF (AOM-injected animals. Arrows indicate sPLA2 stained areas at the crypt base. Images in the dissertation are presented in color.

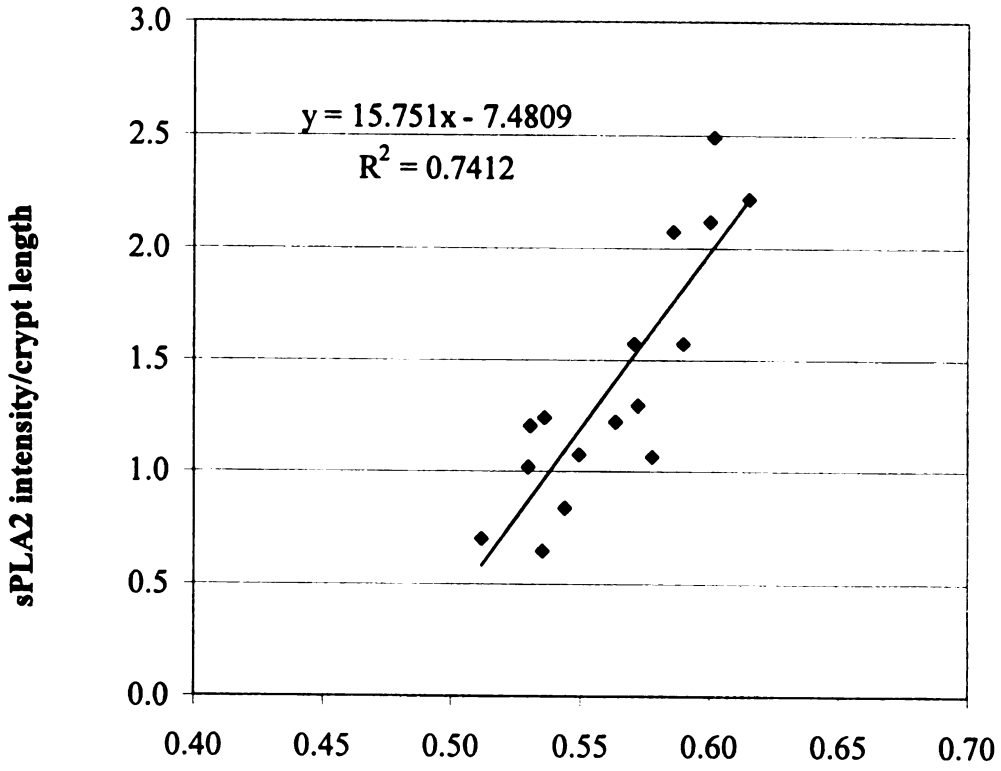


FIGURE 2. Correlation between PCNA proliferation zone (highest $PCNA^+$ cell/crypt height) and sPLA2 immunoreactivity in colonic crypts. Using regression analysis, a highly significant correlation was detected ($r = 0.86$, $P = 0.0001$).

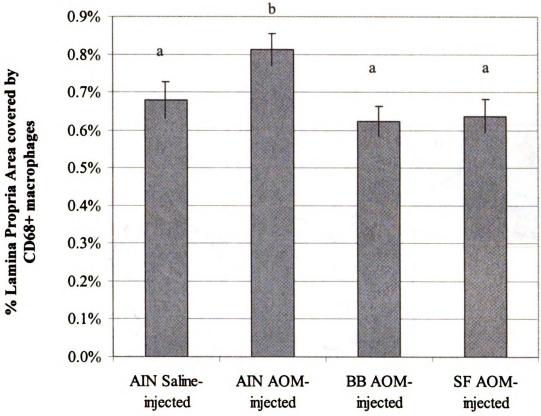


FIGURE 3. Percent area of distal colonic lamina propria covered by CD68 macrophages. Lamina propria and CD68+ areas were quantitated using Bioquant software. Superscripts denote significant differences between groups (P = 0.04). Tumor tissue was used as a positive control (5.4%, data not shown).

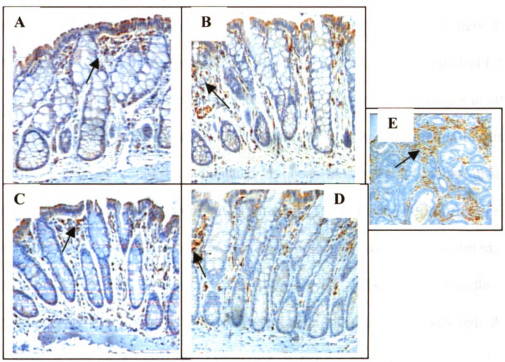


FIGURE 4. Immunoreactivity for C68+ macrophages in colonic mucosa. (A) AIN-saline-injected, (B) AIN(AOM-injected), (C) SF (AOM-injected) and (D) BB(AOM-injected) animals, (E) colon tumor. Arrows indicate CD68+ stained areas. Images in the dissertation are presented in color.

E. DISCUSSION.

Chronic inflammation is strongly associated with colon cancer development, and in previous investigations, sPLA2 was identified as a potential modifier of AOM-induced colon cancer in rats. Specifically, expression of sPLA2 was induced by AOM administration irrespective of dietary treatment, but much less so in bean diets, which also inhibit colon cancer in this model. In the current study, we evaluated sPLA2 in colon tissue by immunohistochemistry to determine if AOM induced changes in sPLA2 were related to alterations in epithelial kinetics and inflammatory responses in rats and whether these changes could be attenuated by bean-feeding. We found that rats administered AOM and fed the control (AIN) diet had a higher zone of proliferative cells in colon crypts than rats fed beans or control (saline-injected) animals. In addition, there was an increase in the proportion of PCNA-labeled cells in the middle crypt compartment. We also detected elevated expression of sPLA2 in AIN-fed, AOM-injected animals and determined that expression of sPLA2 correlated significantly with the proliferative zone, suggesting an inverse relationship between differentiation and sPLA2 immunoreactivity.

Epithelial production of mucin glycoproteins and trefoil peptides as well as tight junction barriers normally constrain exposure of the epithelium and underlying lamina propria to lumenal antigens (28). The localization of sPLA2 within in the lower crypt regions detected in this study and previously by others (24) may be important in transcriptional regulation of the gene, thereby contributing to inflammatory events when mucosal integrity is impaired. The rat gene encoding sPLA2 contains binding sites for the transcription factors CEBP and nuclear factor κ B (NFκB) (29), with the latter often

increased during inflammation and cancer (30). Toll-like receptor 4 (TLR4), also expressed at the crypt base (31), mediates NFκB activation and cytokine release in response to the bacterial antigen lipopolysaccharide (LPS) (32). It has been demonstrated *in vitro* that induction of colonocyte differentiation results in down-regulation of TLR4 receptor mRNA and reduced responsiveness to LPS (33). Although differentiated cells are more exposed to lumenal antigens and therefore would be expected to have lower proinflammatory function, these data would suggest that the ratio of differentiated to undifferentiated colonocytes within the crypt may affect the duration and severity of inflammation invoked following damage to the epithelium or as a result of poor barrier function.

Consistent with this, we also detected significant differences, although modest, in macrophage infiltration in rats treated with AOM and fed the control diet. Resident macrophages underlying the epithelial surface have been proposed to play a role in immediate microbial defenses (34), however monocytes can be recruited to sites of inflammation through local production of chemokines (35). Infiltration of tumor-associated macrophages (TAM) is frequently observed during colon cancer development, and in humans, increases during tumor progression (36). Chronic activation of macrophages has been associated with cancer promotion by producing growth and angiogenic factors and contributing to tissue damage (36). Although changes in the percentage of macrophages were small in this study, a higher presence of macrophages and sPLA2 in AIN-fed, AOM-treated animals compared to bean-fed animals is consistent with enhanced tumorigenesis.

In conclusion, this study demonstrated that bean-based diets, which inhibit experimental colon cancer, also reduce early biomarkers of inflammation in the colonic epithelium. Both black-bean and soy flour-fed animals treated with the carcinogen AOM had lower colonic sPLA2, proliferative indices, and macrophage infiltration into the colonic mucosa as compared to animals fed a casein-based diet. Further, we were able to demonstrate a positive and significant correlation between the levels of sPLA2 within colonic crypts and the proliferative zone. These data suggest that bean-feeding reduces early inflammatory events induced by AOM, which is inversely associated with cellular differentiation.

F. LITERATURE CITED.

1. Ferlay J, Bray F, Pisani P, and Parkin DM. (2002) *GLOBOCAN 2002: Cancer incidence, mortality, and prevalence worldwide,* IARC Press, Lyon, France, http://www-depdb.iarc.fr/globocan/GLOBOframe.htm.

2. American Institute of Cancer Research/World Cancer Research Fund (AICR/WCRF). (1997) Colon, rectum. In *Food, Nutrition and the Prevention of Cancer: A Global Perspective,* BANTA Book Group, Menasha, WI, pp. 216-51.

3. Hughes JS, Ganthavorn C, and Wilson-Sanders S. (1997) Dry beans inhibit azoxymethane-induced colon carcinogenesis in F344 rats. *Journal of Nutrition.* 127: 2328-2333

4. Hangen LA and Bennink MR. (2003) Consumption of black beans and navy beans (Phaseolus vulgaris) reduced axozymethane-induced colon cancer in rats. *Nutrition and Cancer.* 44: *60-6*

5. Bennink MR and Om AS. (1998) Inhibition of colon cancer (CC) by soy phytochemicals but not by soy protein. *Faseb Journal.* 12(5): 3808.

6. Bennink MR, Om AS and Miyagi Y. (1999) Inhibition of colon cancer (CC) by soy flour but not by genistin or a mixture of isoflavones. *Faseb Journal.* 13(4): A50-A50.

7. Bennink MR, Om AS and Miyagi Y. (2000) Inhibition of colon cancer (CC) by wheat bran, soy flour, and flax. *Faseb Journal.* 14(4): A217-A217.

8. Rondini EA and Bennink MR. (2002) Defatted soy flour, but not soy concentrate, reduces azoxymethane-induced colon carcinogenesis. *Journal of Nutrition.* 132(3): 589S-589S.

9. Buckland AG and Wilton DC. (2000) The antibacterial properties of secreted phospholipases A(2). *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids.*1488(1-2): 71-82.

10. Murakami M, Kudo I and Inoue K. (1995) Secretory Phospholipases a(2). *Journal of Lipid Mediators and Cell Signalling.* 12(2-3): 119-130.

11. Zallen G, Moore EE, Johnson JL *et al.* (1998) New mechanisms by which secretory phospholipase A(2) stimulates neutrophils to provoke the release of cytotoxic agents. *Archives of Surgery. 1*33(11): 1229-1232.

12. Baek SH, Kwon TK, Lim Jh *et al.* (2000) Secretory phospholipase A2-potentiated inducible nitric oxide synthase expression by macrophages requires NF-kappa B activation. *J Immunol.* 15:164(12): 6359-65.

13. Baek SH, Yun SS, Kwon TK *et al.* (1999) The effects of two new antagonists of secretory PLA2 on TNF, iNOS, and COX-2 expression in activated macrophages. *Shock.* 12(6): 473-8.

14. Nevalainen TJ, Haapamaki MM and Gronroos JM. (2000) Roles of secretory phospholipases A(2) in inflammatory diseases and trauma. *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids.* 1488(1-2): 83-90.

15. Ogawa M. (1997) Group II phospholipase A(2) in neoplastic diseases. *Phospholipase A2.* 24(200-204).

16. Haapamaki MM, Gronroos JM, Nurmi H *et al.* (1999) Phospholipase A(2) in serum and colonic mucosa in ulcerative colitis. *Scandinavian Journal of Clinical & Laboratory Investigation.* 59(4): 279-287.

17. Partrick DA, Moore EE, Silliman CC *et al.* (2001) Secretory phospholipase A(2) activity correlates with postinjury multiple organ failure. *Critical Care Medicine.* 29(5): 989-993.

18. Graff JR, Konicek BW, Deddens JA *et al.* (2001) Expression of group IIa secretory phospholipase A2 increases with prostate tumor grade. *Clinical Cancer Research.* 7(12): 3857-3861.

19. Niessen HWM, Krijnen PAJ, Visser CA *et al.* (2003) Type II secretory phospholipase A2 in cardiovascular disease: a mediator in atherosclerosis and ischemic damage to cardiomyocytes? *Cardiovascular Research.* 60(1): 68-77.

20. Fahlgren A, Hammarstrom S, Danielsson A and Hammarstrom ML. (2003) Increased expression of antimicrobial peptides and lysozyme in colonic epithelial cells of patients with ulcerative colitis. *Clinical and Experimental Immunology.* 131(1): 90-101.

21. Kennedy BP, Soravia C, Moffat J *et al.* (1998) Overexpression of the nonpancreatic secretory group II PLA(2) messenger RNA and protein in colorectal adenomas from familial adenomatous polyposis patients. *Cancer Research.* 58(3): 500-503.

22. Edhemovic I, Snoj M, Kljun A and Golouh R. (2001) Immunohistochemical localization of group II phospholipase A2 in the tumours and mucosa of the colon and rectum. *European Journal of Surgical Oncology.* 27(6): 545-548.

23. Ikegami T, Matsuzaki Y, Shoda J *et al.* (1996) Ursodeoxycholic acid (UDCA) suppresses enhanced expression of group-II phospholipase-A2 (PLA2) on colonic tissue of azoxymethane (AOM) treated rats. *Hepatology.* 24(4): 1599-1599.

24. Papanikolaou A, Wang QS, Mulherkar R, Bolt A and Rosenberg DW. (2000) Expression analysis of the group IIA secretory phospholipase A(2) in mice with differential susceptibility to azoxymethane-induced colon tumorigenesis. *Carcinogenesis.* 21(2): 133-138.

25. Qiao L, Kozoni V, Tsioulias GJ *et al.* (1995) Selected Eicosanoids Increase the Proliferation Rate of Human Colon-Carcinoma Cell-Lines and Mouse Colonocytes in-Vivo. *Biochimica Et Biophysica Acta-Lipids and Lipid Metabolism.* 1258(2): 215-223.

26. Piazza GA, Rahm ALK, Krutzsch M *et al.* (1995) Antineoplastic Drugs Sulindac Sulfide and Sulfone Inhibit Cell-Growth by Inducing Apoptosis. *Cancer Research.* 55(14): 3110-3116.

27. Boolbol SK, Dannenberg AJ, Chadburn A *et al.* (1996) Cyclooxygenase-2 overexpression and tumor formation are blocked by sulindac in a murine model of familial adenomatous polyposis. *Cancer Research.* 56(11): 2556-2560.

28. Mayer L. (2003) Mucosal immunity. *Pediatrics.* 111(6 Pt 3): 1595-600.

29. Andreani M, Olivier JL, Berenbaum F *et al.* (2000) Transcriptional regulation of inflammatory secreted phospholipases A(2). *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids.* 1488(1-2): 149-158.

30. Karin M and Greten FR. (2005) NF kappa B: Linking inflammation and immunity to cancer development and progression. *Nature Reviews Immunology.* 5(10): 749-759.

31. Ortega-Cava CF, Ishihara S *et al.* (2003) Strategic compartmentalization of Toll-like receptor 4 in the mouse gut. J Immunol. 15;170(8): 3977-85.

32. Yuan Q and Walker WA. (2004) Innate immunity of the gut: Mucosal defense in health and disease. *Journal of Pediatric Gastroenterology and Nutrition.* 38(5): 463-473.

33. Lee SK, Il Kim T, Kim YK *et al.* (2005) Cellular differentiation-induced attenuation of LPS response in HT-29 cells is related to the down-regulation of TLR4 expression. *Biochem Biophys Res Commun.* 18;337(2): 457-63.

34. Gewirtz AT and Madara JL. (2001) Periscope, up! Monitoring microbes in the intestine. *Nature Immunology.* 2(4): 288-290.

35. Balkwill F and Mantovani A. (2001) Inflammation and cancer: back to Virchow? *Lancet.* 357(9255): 539-545.

VI. SUMMARY AND CONCLUSIONS.

In conclusion, using the AOM-induced rodent model, this research provided a molecular basis for epidemiological and experimental observations that beans inhibit colon cancer. In the first two experiments, microarrays were utilized to identify global changes in gene expression contributing to the molecular pathogenesis of AOM-induced colon carcinogenesis in rats. These studies demonstrated that induction of genes involved in inflammation and immune responses are involved in colon cancer progression and some of these genes (sPLA2, NP defensin 3α, MHC II-related genes) appear to explain abnormalities in normal mucosa permissive for tumorigenesis. Feeding bean-based diets, which inhibited tumor formation by 60% in these animals, modulated colonic expression of genes consistent with reduced proliferation (CDC2, cyclin B1, TOP2A, retinoblastoma), higher apoptotic indices (GADD 45α, CEACAM1), reduced innate immune responsiveness (defensin 3α, sPLA2, TLR4), and alterations in extracellular matrix components (fibronectin 1, collagen 1α1). More specifically, bean-feeding suppressed carcinogen-induced upregulation of defensin 3α, sPLA2, fibronectin, and collagen 1α1. These data implied an association between genes involved in crypt cytokinetics and inflammation to future colon cancer development. Therefore in the third experiment, immunohistochemical techniques were employed to assess dietary differences in proliferation indices, sPLA2, and macrophage infiltration during AOM-induced colon carcinogenesis. It was demonstrated that bean-feeding reduced carcinogen-induced alterations in the percentage of proliferating cells in the middle crypt compartment, the proliferation zone, the amount of sPLA2 protein, and moderately reduced infiltration of macrophages in colonic mucosa. Further, a positive correlation was found between the proliferation zone and immunoreactivity for sPLA2.

Collectively, microarray and histochemical data imply that bean-diets inhibit tumorigenesis by helping maintain a microanatomical "niche" within colon crypts. Restraining the expansion of proliferative cells towards the lumen is closely related to lower expression of innate defense, inflammatory, and extracellular matrix genes more characteristic of crypt progenitor cells. Dietary agents that increase the proportion of differentiated cells and/or reinforce the mucosal barrier may reduce exposure of proliferative cells to lumenal contents and thereby limit bacterial-induced inflammation.

VII. FUTURE DIRECTIONS.

Colon cancer is the fourth most common cancer and second leading cause of cancer mortality in the United States. Although colon cancer, if detected at an early stage, has a good prognosis, education and accessibility to health care are barriers to screening in the general population and therefore only 39% of cancers are detected this early. Dietary habits are strongly associated with cancer risk, and this research lends further support to epidemiological and experimental data that consumption of bean-based diets inhibits colon cancer development. The finding that beans reduce early biomarkers of colonic inflammation is also consistent with the inverse association of long-term NSAID use on CRC risk. By demonstrating that diet can modulate genes in normal mucosa permissive for tumorigenesis provides a rationale basis for clinical trials to determine the true potential of beans to prevent colon cancer in humans. Further, it is well recognized that individuals previously treated for colon cancer are at a 30% risk of recurrence of the disease. This observation, termed a "field defect" or "field cancerization" is proposed to be related to molecular abnormalities in areas surrounding cancer tissue. Results form this study suggest that some of these abnormalities are related to changes in cytokinetic, inflammatory, and extracellular matrix components that alter differentiation programming along the colonic crypt. Confirming some of these gene changes in humans, further identifying what causes these changes to occur, and determining if bean consumption can reverse these changes would strengthen the relationship of bean consumption on colon cancer inhibition and may provide useful adjunct therapy for those at risk.

VIII. APPENDICES.

SUPPLEMENTARY TABLE 1. Supplemental list of global genetic alterations in AOM-induced colon cancers relative to normal colonic mucosa. [1]

Gene symbol	Gene Title	Tumor
I. Cell adhesion, cell communication		
Cd44	CD44 antigen	13
Lgals1	lectin, galactose binding, soluble 1	13
Icam1	intercellular adhesion molecule 1	3.3
Itga1	integrin alpha 1	3.0
Gpc1	glypican 1	2.5
Alcam	activated leukocyte cell adhesion molecule	2.2
Gjb1	gap junction membrane channel protein beta 1	0.5
Cldn3	claudin 3	0.5
Ceacam10	CEA-related cell adhesion molecule 10	0.3
Thbs4	thrombospondin 4	0.2
II. Cytoskeleton, microtubule processes, structural filaments		
Tuba1	alpha-tubulin	9.6
Cnn3	calponin 3, acidic	6.8
Tagln	transgelin	6.4
Vim	vimentin	6.1
Actg2	actin, gamma 2	5.7
---	similar to tubulin, beta (predicted)	4.2
Pls3	plastin 3 (t-isoform)	3.2
Stmn1	stathmin 1	2.9
---	similar to type xv collagen (loc298069), mrna	2.8
Krt1-18	keratin complex 1, acidic, gene 18	2.4
Cnp1	Cyclic nucleotide phosphodiesterase 1	2.3
Tctex1	t-complex testis expressed 1	2.3
Tpm1	tropomyosin 1, alpha	2.3
Cfl1	cofilin 1	2.2
Pdlim3	actinin alpha 2 associated lim protein	2.2
Myo1b	myosin ib	2.0
Tubb5	tubulin, beta 5	2.0

SUPPLEMENTARY TABLE 1. Supplemental list of global genetic alterations in AOM-induced colon cancers relative to normal colonic mucosa. *(continued)* [1]

Gene symbol	Gene Title	Tumor
II. Cytoskeleton, microtubule processes, structural filaments		
---	myosin regulatory light chain	2.0
Myo1a	myosin, heavy polypeptide-like	0.4
Krt21	cytokeratin 21	0.2
---	similar to actinin, alpha 2 (loc291245), mrna	0.1
III. Electron transport, oxidoreductase, detoxification		
Srd5a2	steroid 5-alpha-reductase 2	11
---	udp glycosyltransferase 1 family polypeptide a2	4.3
Mgst1	microsomal glutathione s-transferase 1	4.0
Gpx2	glutathione peroxidase 2	3.4
Cybb	endothelial type gp91-phox gene	3.3
Xdh	xanthine dehydrogenase	2.9
Prdx1	peroxiredoxin 1	2.6
Nqo1	nad(p)h dehydrogenase, quinone 1	2.3
Gstm5	glutathione s-transferase, mu 5	2.2
---	metallothionein	2.2
Pam	peptidylglycine alpha-amidating monooxygenase	0.5
Cyb5	cytochrome b5	0.5
Cyp2j9	cytochrome p450 monooxygenase	0.5
Cbr1	carbonyl reductase 1	0.5
Tst	thiosulfate sulfurtransferase	0.5
---	udp glycosyltransferase 1 family, polypeptide a6	0.4
Akr7a3	aldo-keto reductase family 7, member A3 (aflatoxin aldehyde reductase)	0.4
Nat1	n-acetyltransferase 1 (arylamine n-acetyltransferase)	0.4
Cyp3a13	cytochrome p450 3a9	0.4
Gstm1	glutathione s-transferase, mu 1	0.2
Nat2	n-acetyltransferase-2	0.2
Cyp2d10	cytochrome p450 2d1	0.1

SUPPLEMENTARY TABLE 1. Supplemental list of global genetic alterations in AOM-induced colon cancers relative to normal colonic mucosa. ***(continued)*** [1]

Gene symbol	Gene Title	Tumor
IV. Enzymes, other		
Tgm1	transglutaminase 1	24
---	protease, serine, 22 (predicted)	6.1
---	lysyl oxidase	5.4
Pde4b	phosphodiesterase 4b	5.1
Ppap2a	phosphatidate phosphohydrolase type 2a	3.6
---	Pctaire2	3.3
Apex1	apurinic/apyrimidinic endonuclease 1	3.2
---	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 1	3.1
Siat1	sialyltransferase 1	2.7
Pafah1b2	platelet-activating factor acetylhydrolase alpha 2 subunit (paf-ah alpha 2)	2.3
---	TC10-like Rho GTPase	2.2
Prmt3	protein arginine n-methyltransferase 3(hnrnp methyltransferase s. cerevisiae)-like 3	2.2
---	protein kinase N3 (predicted)	2.1
---	phenylalanine-tRNA synthetase-like, alpha subunit (predicted)	2.0
Galnt5	udp-galnac:polypeptide n-acetylgalactosaminyltransferase t5	0.5
Fut1	fucosyltransferase 1	0.5
Ctbs	di-n-acetylchitobiase	0.5
Uae1	udp-n-acetylglucosamine-2-epimerase/n-acetylmannosamine kinase	0.5
Ckmt1	creatine kinase, mitochondrial 1, ubiquitous	0.4
Gda	guanine deaminase	0.4
Bpnt1	3(2),5-bisphosphate nucleotidase	0.4
---	dimethylarginine dimethylaminohydrolase 1	0.4
Fthfd	10-formyltetrahydrofolate dehydrogenase	0.3
Ampd3	adenosine monophosphate deaminase 3	0.2
Cd38	CD38 antigen	0.2
Ca4	carbonic anhydrase 4	0.2

SUPPLEMENTARY TABLE 1. Supplemental list of global genetic alterations in AOM-induced colon cancers relative to normal colonic mucosa. *(continued)* [1]

Gene symbol	Gene Title	Tumor
V. Other		
---	Thymus cell antigen 1, theta	28
Mgp	matrix gla protein	16
Hspb1	heat shock 27kda protein 1	12
Gbp2	guanylate binding protein 2, interferon-inducible	11
Reg3g	pancreatitis-associated protein 3	8.6
---	S77900 myosin regulatory light chain isoform C [rats, Sprague-Dawley, new-born, heart ventricle, mRNA, 1008 nt]	8.3
Serpinh1	serine (or cysteine) proteinase inhibitor, clade h, member 1	7.5
---	similar to nedd4 ww binding protein 4 (loc311676), mrna	6.8
Calca	calcitonin/calcitonin-related polypeptide, alpha	6.4
Serpine1	serine (or cysteine) proteinase inhibitor, member 1	6.1
Serping1	serine (or cysteine) proteinase inhibitor, clade g (c1 inhibitor), member 1, (angioedema, hereditary)	5.5
Abi2	abl-interactor 2	5.4
---	interferon induced transmembrane protein 3-like	5.2
Lgals3bp	peptidylprolyl isomerase c-associated protein	4.4
Caps	ca2+-dependent activator protein	4.4
Rcn2	reticulocalbin 2	4.2
---	similar to g0s2-like protein (loc289388), mrna	4.0
Nup54	nucleoporin p54	3.5
---	centaurin, beta 1 (predicted)	3.5
S100a4	s100 calcium-binding protein a4	3.4
Anxa6	annexin vi	3.1
Eif2b3	eukaryotic translation initiation factor 2b, subunit 3 (gamma, 58kd)	2.9
LOC257646	ferm-domain-containing protein 163scii	2.9
---	similar to deoxycytidyl transferase (loc316344), mrna	2.7
Hmga1	high mobility group at-hook 1	2.7
Emd	emerin	2.7

SUPPLEMENTARY TABLE 1. Supplemental list of global genetic alterations in AOM-induced colon cancers relative to normal colonic mucosa. *(continued)* [1]

Gene symbol	Gene Title	Tumor
V. Other		
Pmf31	pmf32 protein	2.6
Anxa5	annexin 5	2.6
---	immediate early response 3	2.5
---	similar to myelin protein zero-like 1; protein zero related (loc360871), mrna	2.4
Ehd4	pincher	2.4
---	vacuole membrane protein 1	2.4
Hspca	heat shock protein 1, alpha	2.4
---	similar to mcdc47 (loc288532), mrna	2.3
PVR	tumor-associated antigen 1	2.3
---	notch1-induced protein	2.3
Nudc	nuclear distribution gene c homolog (aspergillus)	2.2
Sepw1	selenoprotein w, muscle 1	2.1
Eef1a1	eukaryotic translation elongation factor 1 alpha 1	2.1
Serpinb5	serine (or cysteine) proteinase inhibitor, clade b, member 5	2.1
Anxa2	annexin A2	2.0
---	heat shock 90kDa protein 1, beta	2.0
Stip1	stress-induced-phosphoprotein 1 (hsp70/hsp90-organizing protein)	2.0
---	similar to expressed sequence au040575 (loc288003), mrna	2.0
Cct3	chaperonin containing TCP1, subunit 3 (gamma)	2.0
---	slo-interacting ankyrin-containing protein	0.5
Kai1	kangai 1	0.5
Resp18	regulated endocrine-specific protein 18	0.5
---	similar to mitochondrial ribosomal protein s6 (loc288253), mrna	0.4
Nmu	neuromedin	0.4
Muc3	mucin 3	0.4
Tgoln2	trans-golgi network protein 1	0.3
Insig1	insulin induced gene 1	0.3

SUPPLEMENTARY TABLE 1. Supplemental list of global genetic alterations in AOM-induced colon cancers relative to normal colonic mucosa. *(continued)* [1]

Gene symbol	Gene Title	Tumor
V. Other		
Unc119	unc-119 homolog (c. elegans)	0.3
Calb3	calbindin 3	0.3
---	z29072cds rnmucinr r.norvegicus (sprague dawley) mrna for mucin	0.2
---	endothelin 2	0.2
Gp2	secretory (zymogen) granule membrane glycoprotein gp2	0.2
---	m81920 ratintmuc rat mucin-like protein mrna	0.1
VI. RNA processing		
Hnrpa1	heterogeneous nuclear ribonucleoprotein a1	3.9
Hrmt1l2	heterogeneous nuclear ribonucleoproteins methyltransferase-like 2 (s. cerevisiae)	3.4
SMN1	survival motor neuron	3.3
Ssb	sjogren syndrome antigen b	2.3
Apobec1	apolipoprotein b editing complex 1	0.3
VII. Signal Transduction		
---	tumor-associated calcium signal transducer 2	31
Pdgfra	platelet derived growth factor receptor, alpha polypeptide	8.3
Fstl1	follistatin-like	8.0
Ptprd	protein tyrosine phosphatase, receptor type, d	7.9
Tnfrsf11b	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	6.4
Axin2	axin2	5.6
Argbp2	arg/abl-interacting protein argbp2	5.4
Ednrb	endothelin receptor type b	4.7
Ptprc	protein tyrosine phosphatase, receptor type, c	4.1
Ltbp1	lanc (bacterial lantibiotic synthetase component c)-like 1	3.7
---	mitogen activated protein kinase kinase kinase kinase 1 (predicted)	3.6
Ptpro	protein tyrosine phosphatase, receptor type, o	3.4
Stk39	serine threonine kinase 39 (ste20/sps1 homolog, yeast)	3.1

SUPPLEMENTARY TABLE 1. Supplemental list of global genetic alterations in AOM-induced colon cancers relative to normal colonic mucosa. *(continued)* [1]

Gene symbol	Gene Title	Tumor
VII. Signal Transduction		
Ptpre	protein tyrosine phosphatase, receptor type, epsilon polypeptide	3.1
Prkg2	protein kinase, cgmp- dependent, type ii	2.5
Arrb2	arrestin, beta 2	2.2
Csrp2	cysteine-rich protein 2	2.2
Map2k6	mitogen-activated protein kinase kinase 6	0.5
Add3	adducin 3, gamma	0.5
---	protein tyrosine phosphatase, non-receptor type 18 (predicted)	0.5
Viprl	vasoactive intestinal peptide receptor 1	0.5
Glgl	selectin, endothelial cell, ligand	0.4
Chnl	chimerin (chimaerin) 1	0.4
Syngr2	synaptogyrin 2	0.4
Egf	epidermal growth factor	0.4
Itpka	inositol 1,4,5-triphosphate 3-kinase	0.3
Prkca	protein kinase c, alpha	0.3
---	tumor necrosis factor (ligand) superfamily, member 13 (predicted)	0.3
Guca2a	guanylate cyclase activator 2a	0.3
Csrp2	cysteine and glycine-rich protein 2	0.3
Duspl	protein tyrosine phosphatase, non-receptor type 16	0.3
Bmp2	bone morphogenetic protein 2	0.2
Pde2a	phosphodiesterase 2a, cgmp-stimulated	0.2
Pde5a	phosphodiesterase 5a, cgmp-specific	0.2
Adora2b	adenosine a2b receptor	0.2
Ndrg2	n-myc downstream-regulated gene 2	0.2
---	bone morphogenetic protein 3	0.1
Plcdl	phospholipase c, delta 1	0.1
Rasgrpl	ras guanyl releasing protein 1	0.1
Sgk	serum/glucocorticoid regulated kinase	0.1

SUPPLEMENTARY TABLE 1. Supplemental list of global genetic alterations in AOM-induced colon cancers relative to normal colonic mucosa. ***(continued)*** [1]

Gene symbol	Gene Title	Tumor
VIII. Transcription regulation, nucleic acid binding		
Msg1	melanocyte-specific gene 1 protein	7.5
Ascl2	achaete-scute complex homolog-like 2 (drosophila)	6.3
Basp1	brain abundant, membrane attached signal protein 1	5.9
Id3	inhibitor of dna binding 3, dominant negative helix-loop-helix protein	5.3
Lhx2	lim homeobox protein 2	4.7
Cebpd	CCAAT/enhancer binding protein (C/EBP), delta	2.8
Nr4a3	nuclear receptor subfamily 4, group a, member 3	2.5
---	transcription factor E2a	2.5
Notch1	notch gene homolog 1, (drosophila)	2.5
Ruvbl1	ruvb-like protein 1	2.4
---	nucleolin	2.3
Fhl2	four and a half lim domains 2	2.2
---	tbp-interacting protein 120a	2.2
Mcmd6	mini chromosome maintenance deficient 6 (s. cerevisiae)	2.1
U83883	p105 coactivator	2.0
Ratireb	iron-responsive element-binding protein	0.5
Nr1d1	nuclear receptor subfamily 1, group d, member 1	0.5
Arntl	aryl hydrocarbon receptor nuclear translocator-like	0.5
Vdr	vitamin d receptor	0.4
Egr2	early growth response 2	0.4
Foxq1	hnf-3/forkhead homolog-1	0.4
Nr4a2	nuclear receptor subfamily 4, group a, member 2	0.4
Nr3c2	nuclear receptor subfamily 3, group c, member 2	0.3
Klf4	kruppel-like factor 4 (gut)	0.3
Thrb	thyroid hormone receptor beta	0.3
Nr1h4	nuclear receptor subfamily 1, group h, member 4	0.2
Pdx1	synaptotagmin 4	0.1

SUPPLEMENTARY TABLE 1. Supplemental list of global genetic alterations in AOM-induced colon cancers relative to normal colonic mucosa. *(continued)* [1]

Gene symbol	Gene Title	Tumor
IX. EST, function unknown		
---	similar to type i hair keratin 6 (loc287698), mrna	54
---	Rat mixed-tissue library Rattus norvegicus cDNA clone rx00304 3', mRNA sequence [Rattus norvegicus]	36
---	transcribed sequences	18
---	rc_AA875531 UI-R-E0-cv-f-12-0-UI.s1 Rattus norvegicus cDNA, 3' end /clone=UI-R-E0-cv-f-12-0-UI /clone_end=3' /gb=AA875531 /gi=2980479 /ug=Rn.256 /len=433	10
---	similar to osteoblast specific factor 2 precursor (loc361945), mrna	9.9
Tmeff1	Rat mixed-tissue library Rattus norvegicus cDNA clone rx00133 3', mRNA sequence [Rattus norvegicus]	8.8
---	transcribed sequences	8.2
---	similar to cornifin alpha (small proline-rich protein 1) (sprr1) (loc365848), mrna	6.2
---	similar to riken cdna 6720467c03 (loc297903), mrna	5.9
---	similar to traf2 and nck interacting kinase, splice variant 4 (loc301363), mrna	5.9
---	transcribed sequences	5.9
---	transcribed sequences	5.4
---	transcribed sequences	4.6
---	similar to loxl protein (loc315714), mrna	4.5
dd25	hypothetical protein	4.4
---	similar to ras association domain family 2 (loc311437), mrna	4.1
---	transcribed sequence with strong similarity to protein sp:p00722 (e. coli) bgal_ecoli beta-galactosidase	4.1
---	similar to riken cdna c730007l20 gene (loc364396), mrna	3.8
---	similar to phospholipid scramblase 3 (loc360549), mrna	3.8
---	similar to capg protein (loc297339), mrna	3.7
---	transcribed sequence with moderate similarity to protein sp:p00722 (e. coli) bgal_ecoli beta-galactosidase	3.4

SUPPLEMENTARY TABLE 1. Supplemental list of global genetic alterations in AOM-induced colon cancers relative to normal colonic mucosa. *(continued)* [1]

Gene symbol	Gene Title	Tumor
IX. EST, function unknown		
---	m91234 rat vl30 element mrna /cds=unknown /gb=m91234 /gi=207671 /ug=rn.18005 /len=1132	3.4
---	similar to ab2-008 (loc290270), mrna	3.3
---	similar to plakophilin 4 (p0071) (loc295625), mrna	3.3
---	small proline-rich protein gene	3.3
---	transcribed sequence with moderate similarity to protein sp:p00722 (e. coli) bgal_ecoli beta-galactosidase	3.2
---	transcribed sequences	3.1
---	dd3g2-3 mrna, partial sequence	3.1
---	loc363015 (loc363015), mrna	3.1
---	similar to chromosome 14 open reading frame 50 (loc299153), mrna	3.1
---	Rat mixed-tissue library Rattus norvegicus cDNA clone rx01696 3', mRNA sequence [Rattus norvegicus]	2.9
---	transcribed sequences	2.8
---	x62951mrna rnpbus19 r.norvegicus mrna (pbus19) with repetitive elements	2.7
---	transcribed sequences	2.6
---	similar to jtv1-pending protein (loc288480), mrna	2.5
---	Similar to T-cell receptor beta-chain	2.5
---	transcribed sequences	2.5
---	similar to riken cdna 6720485c15 (loc360639), mrna	2.5
---	similar to riken cdna 1500011h22 (loc288667), mrna	2.3
---	transcribed sequences	2.3
---	similar to phosphoprotein enriched in astrocytes 15 (loc364052), mrna	2.3
---	transcribed sequences	2.3
---	Rat mixed-tissue library Rattus norvegicus cDNA clone rx01427 3', mRNA sequence [Rattus norvegicus]	2.2
---	x05472cds#3 rnrep24r rat 2.4 kb repeat dna right terminal region	2.2

SUPPLEMENTARY TABLE 1. Supplemental list of global genetic alterations in AOM-induced colon cancers relative to normal colonic mucosa. *(continued)* [1]

Gene symbol	Gene Title	Tumor
IX. EST, function unknown		
---	similar to hypothetical protein flj14360 (loc303792), mrna	2.2
---	x53581cds#3 rnlined r.norvegicus long interspersed repetitive dna containing 7 orf's	2.2
---	similar to 25 kda fk506-binding protein (loc299104), mrna	2.2
---	similar to hypothetical protein d15wsu59e (loc294810), mrna	2.2
---	transcribed sequence with moderate similarity to protein ref:np_036191.1 (m.musculus) ca<2+>dependent activator	2.1
---	similar to fksg27 (loc292708), mrna	2.1
---	sideroflexin 1 (predicted)	2.1
---	similar to n-acetylglucosamine-6-sulfatase precursor (g6s) (glucosamine-6-sulfatase) (loc299825), mrna	2.1
---	similar to ga17 protein (loc295975), mrna	2.1
---	/clone=RHEAC15 /clone_end=3' /gb=AA799526 /gi=2862481 /ug=Rn.6351 /len=626	2.0
---	similar to myosin id (myosin heavy chain myr 4) (loc289785), mrna	2.0
---	transcribed sequences	2.0
---	similar to aspartyl aminopeptidase (loc301529), mrna	2.0
---	transcribed sequences	2.0
---	transcribed sequences	0.5
MGC72616	unknown (protein for mgc:72616)	0.5
---	similar to bc002216 protein (loc313771), mrna	0.5
---	transcribed sequence with moderate similarity to protein sp:q13576 (h.sapiens) iqg2_human ras gtpase-activating-like	0.5
---	liver regeneration-related protein lrrg07 mrna, complete cds	0.5
---	similar to ms4a8b protein (loc361733), mrna	0.5
---	similar to riken cdna 2700099c19 (loc302422), mrna	0.5
---	Similar to KIAA1592 protein	0.5
---	transcribed sequences	0.5
---	transcribed sequences	0.4

SUPPLEMENTARY TABLE 1. Supplemental list of global genetic alterations in AOM-induced colon cancers relative to normal colonic mucosa. *(continued)* [1]

Gene symbol	Gene Title	Tumor
IX. EST, function unknown		
---	similar to mitogen-inducible gene 6 protein homolog (mig-6) (gene 33 polypeptide) (loc313729), mrna	0.4
---	hypothetical loc290595 (loc290595), mrna	0.4
---	transcribed sequence with strong similarity to protein sp:q15029 (h.sapiens) u5s1_human 116 kda u5 small nuclear ribonucleoprotein component	0.4
---	x07266cds rrg33a rat mrna for gene 33 polypeptide	0.4
---	similar to bai1-associated protein 1; ww domain-containing protein 3; atrophin-1 interacting protein 3 (loc297463), mrna	0.4
---	transcribed sequences	0.3
---	transcribed sequences	0.3
---	similar to hypothetical protein MGC15606	0.3
---	rc_AA892248 EST196051 Rattus norvegicus cDNA, 3' end /clone=RKIAO18 /clone_end=3' /gb=AA892248 /gi=3019127 /ug=Rn.2277 /len=587	0.3
---	Rat mixed-tissue library Rattus norvegicus cDNA clone rx04959 3', mRNA sequence [Rattus norvegicus]	0.2
---	transcribed sequences	0.2
---	rc_AA892248 EST196051 Rattus norvegicus cDNA, 3' end /clone=RKIAO18 /clone_end=3' /gb=AA892248 /gi=3019127 /ug=Rn.2277 /len=587	0.2
---	similar to atp sulfurylase/aps kinase 2 (loc294103), mrna	0.1
---	Rat mixed-tissue library Rattus norvegicus cDNA clone rx01462 3', mRNA sequence [Rattus norvegicus]	0.1

[1] Data expressed as mean-fold change differences in tumor tissue relative to values for non-neoplastic colon mucosa (n = 2-4/group).

SUPPLEMENTARY TABLE 2. Supplemental list of genes differentially affected by AOM treatment in distal colonic epithelium of male F344 rats. [1]

Gene symbol	Common	Saline-Injected	AOM-Injected
I. Cell cycle, cell growth and maintenance, apoptosis			
Cd53	cd53 antigen	1.0 [a]	2.2 [b]
---	clusterin	1.0 [a]	1.8 [b]
Sh3kbp1	sh3-domain kinase binding protein 1	1.0 [a]	1.5 [b]
Ns	nucleostemin	1.0 [a]	1.4 [b]
E2f5	e2f transcription factor 5	1.0 [a]	1.3 [b]
c-fos	c-fos oncogene	1.0 [b]	0.7 [a]
Apbb3	amyloid beta (a4) precursor protein-binding, family b, member 3	1.0 [b]	0.7 [a]
Kitl	kit ligand	1.0 [b]	0.7 [a]
Jun	v-jun sarcoma virus 17 oncogene homolog (avian)	1.0 [b]	0.6 [a]
Camk2d	calcium/calmodulin-dependent protein kinase ii, delta	1.0 [b]	0.6 [a]
II. Channel, transporters, & carriers			
Apoe	apolipoprotein e	1.0 [a]	1.3 [b]
Slc3a2	solute carrier family 3, member 2	1.0 [a]	1.3 [b]
Slc4a1	solute carrier family 4, member 1	1.0 [a]	1.2 [b]
Grina	nmda receptor glutamate-binding chain	1.0 [b]	0.7 [a]
Slc9a2	solute carrier family 9, member 2	1.0 [b]	0.7 [a]
Atp1b1	atpase na+/k+ transporting beta 1 polypeptide	1.0 [b]	0.7 [a]
III. Electron transport, oxidoreductase, detoxification			
Gpx2	glutathione peroxidase 2	1.0 [a]	1.3 [b]
Cbr1	carbonyl reductase 1	1.0 [b]	0.7 [a]
Dia1	diaphorase 1	1.0 [b]	0.7 [a]
Cyp2j9	cytochrome p450 monooxygenase	1.0 [b]	0.7 [a]
IV. Energy metabolism			
Me1	malic enzyme 1	1.0 [a]	1.4 [b]
Padi2	peptidyl arginine deiminase, type 2	1.0 [a]	1.4 [b]
Odc1	ornithine decarboxylase 1	1.0 [a]	1.3 [b]

SUPPLEMENTARY TABLE 2. Supplemental list of genes differentially affected by AOM treatment in distal colonic epithelium of male F344 rats. *(continued)* [1]

Gene symbol	Common	Saline-Injected	AOM-Injected
IV. Energy metabolism			
Fdft1	farnesyl diphosphate farnesyl transferase 1	1.0 [a]	1.3 [b]
Ehhadh	enoyl-coenzyme a, hydratase/3-hydroxyacyl coenzyme a dehydrogenase	1.0 [b]	0.7 [a]
Cdo1	cytosolic cysteine dioxygenase 1	1.0 [b]	0.7 [a]
Pygb	brain glycogen phosphorylase	1.0 [b]	0.7 [a]
V. Enzymes, other			
LOC64300	c1-tetrahydrofolate synthase	1.0 [a]	1.4 [b]
Rab6	rab6, member ras oncogene family	1.0 [a]	1.3 [b]
Ca4	carbonic anhydrase 4	1.0 [b]	0.7 [a]
Ppm1b	protein phosphatase 1b, magnesium dependent, beta isoform	1.0 [b]	0.7 [a]
Nudt4	diphosphoinositol polyphosphate phosphohydolase type ii	1.0 [b]	0.7 [a]
Comt	catechol-o-methyltransferase	1.0 [b]	0.7 [a]
Nat1	n-acetyltransferase-2	1.0 [b]	0.6 [a]
Inpp4a	inositol polyphosphate-4-phosphatase, type 1	1.0 [b]	0.5 [a]
VI. Nucleic acid binding, transcription regulation			
Basp1	brain acidic membrane protein	1.0 [a]	1.5 [b]
Hmgb2	high mobility group box 2	1.0 [a]	1.4 [b]
Hnrpa1	heterogeneous nuclear ribonucleoprotein a1	1.0 [a]	1.4 [b]
Nfia	nuclear factor i/a	1.0 [a]	1.4 [b]
Gtf2f2	general transcription factor iif, polypeptide 2 (30kd subunit)	1.0 [a]	1.3 [b]
Npm1	nucleophosmin 1	1.0 [a]	1.3 [b]
Klf4	kruppel-like factor 4 (gut)	1.0 [b]	0.7 [a]
Nr5a2	nuclear receptor subfamily 5, group a, member 2	1.0 [b]	0.7 [a]
Hnf4a	hepatocyte nuclear factor 4, alpha	1.0 [b]	0.7 [a]

SUPPLEMENTARY TABLE 2. Supplemental list of genes differentially affected by AOM treatment in distal colonic epithelium of male F344 rats. *(continued)* [1]

Gene symbol	Common	Saline-Injected	AOM-Injected
VII. Signal transduction			
Pde4b	phosphodiesterase 4b	1.0 [a]	1.4 [b]
Notch3	Notch 3	1.0 [a]	1.4 [b]
Aps	adaptor protein with pleckstrin homology and src homology 2 domains	1.0 [a]	1.3 [b]
Cxcr4	chemokine (C-X-C motif) receptor 4	1.0 [a]	1.3 [b]
Nmu	neuromedin	1.0 [a]	1.3 [b]
Oprs1	opioid receptor, sigma 1	1.0 [a]	1.3 [b]
Rgs19	calcium/calmodulin-dependent protein kinase I	1.0 [a]	1.3 [b]
Cd81	CD 81 antigen	1.0 [a]	1.2 [b]
Sstr2	somatostatin receptor 2	1.0 [b]	0.7 [a]
Erbb3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	1.0 [b]	0.7 [a]
Pld1	phospholipase D1	1.0 [b]	0.7 [a]
Plcd1	phospholipase C, delta 1	1.0 [b]	0.7 [a]
Kras2	Kirsten rat sarcoma viral oncogene homologue 2 (active)	1.0 [b]	0.7 [a]
----	adrenergic receptor kinase, beta 1	1.0 [b]	0.7 [a]
Rabep2	rabaptin, RAB GTPase binding effector protein 2	1.0 [b]	0.7 [a]
Dusp1	dual specificity phosphatase 1	1.0 [b]	0.6 [a]
VIII. Other			
Ctf1	cardiotrophin 1	1.0 [a]	1.5 [b]
Loc192269	pr-et2 encoded oncodevelopmental protein	1.0 [a]	1.4 [b]
U83883	p105 coactivator	1.0 [a]	1.4 [b]
Dnm1l	dynamin 1-like	1.0 [a]	1.4 [b]
Xrcc5	x-ray repair complementing defective repair in chinese hamster cells 5	1.0 [a]	1.4 [b]
Pex11a	peroxisomal membrane protein pmp26p (peroxin-11)	1.0 [a]	1.4 [b]
Lgals3bp	peptidylprolyl isomerase c-associated protein	1.0 [a]	1.4 [b]

SUPPLEMENTARY TABLE 2. Supplemental list of genes differentially affected by AOM treatment in distal colonic epithelium of male F344 rats. *(continued)* [1]

Gene symbol	Common	Saline-Injected	AOM-Injected
VIII. Other			
Rcn2	reticulocalbin 2	1.0 [a]	1.4 [b]
Napl1l	nucleosome assembly protein 1-like 1	1.0 [a]	1.3 [b]
p47	p47 protein	1.0 [a]	1.3 [b]
SMN1	survival motor neuron	1.0 [a]	1.3 [b]
Ssr3	trap-complex gamma subunit	1.0 [a]	1.3 [b]
Nolc1	nucleolar phosphoprotein p130	1.0 [a]	1.3 [b]
Apbb3	cysteine string protein	1.0 [b]	0.7 [a]
Grina	endothelin 2	1.0 [b]	0.6 [a]
IX. EST, function unclassified			
----	transcribed sequence with weak similarity to protein ref:np_079050.1 (h.sapiens) hypothetical protein flj21924 [homo sapiens]	1.0 [a]	1.8 [b]
----	similar to myosin id (myosin heavy chain myr 4) (loc289785), mrna	1.0 [a]	1.6 [b]
----	transcribed sequence with weak similarity to protein ref:np_006410.1 (h.sapiens) small inducible cytokine b subfamily	1.0 [a]	1.5 [b]
----	similar to riken cdna 2610528e23 (loc288176), mrna	1.0 [a]	1.5 [b]
----	similar to talin (loc313494), mrna	1.0 [a]	1.4 [b]
----	rc_h31217 est105044 rattus norvegicus cdna, 3' end /clone=rpcaf34 /clone_end=3' /gb=h31217	1.0 [a]	1.4 [b]
----	similar to nadp+-specific isocitrate dehydrogenase (loc293043), mrna	1.0 [a]	1.4 [b]
----	similar to dna directed rna polymerase ii polypeptide i; polymerase (rna) ii (dna directed) polypeptide i (14.5kd); dna directed rna polymerase ii 14.5 kda polypeptide (loc292778), mrna	1.0 [a]	1.4 [b]

SUPPLEMENTARY TABLE 2. Supplemental list of genes differentially affected by AOM treatment in distal colonic epithelium of male F344 rats. *(continued)* [1]

Gene symbol	Common	Saline-Injected	AOM-Injected
IX. EST, function unclassified			
----	rc_aa892400 est196203 rattus norvegicus cdna, 3' end /clone=rkiaq01 /clone_end=3' /gb=aa892400 /gi=3019279 /ug=rn.14755 /len=394	1.0 [a]	1.4 [b]
----	transcribed sequence with strong similarity to protein ref:np_036526.2 (h.sapiens) prefoldin 2 [homo sapiens]	1.0 [a]	1.4 [b]
----	similar to riken cdna 1110032a17 (loc362156), mrna	1.0 [a]	1.4 [b]
----	similar to cul2 protein (loc361258), mrna	1.0 [a]	1.3 [b]
----	dd6a4-2(5) mrna, partial sequence	1.0 [a]	1.3 [b]
----	similar to riken cdna 3110052n05 (loc361351), mrna	1.0 [a]	1.3 [b]
----	transcribed sequences	1.0 [a]	1.3 [b]
----	similar to 60s ribosomal protein l3-like (loc287122), mrna	1.0 [a]	1.3 [b]
----	rc_h31128 est104855 rattus norvegicus cdna, 3' end /clone=rpcad04 /clone_end=3' /gb=h31128 /gi=976550 /ug=rn.7211 /len=308	1.0 [a]	1.3 [b]
----	transcribed sequences	1.0 [a]	1.3 [b]
----	similar to riken cdna 2900010m23 (loc361805), mrna	1.0 [a]	1.3 [b]
----	similar to interferon regulatory factor 7 (loc293624)	1.0 [a]	1.3 [b]
----	transcribed sequences	1.0 [a]	1.3 [b]
----	transcribed sequence with moderate similarity to protein pdb:1lbg (e. coli) b chain b, lactose operon repressor bound to 21-base pair symmetric operator dna, alpha carbons only	1.0 [a]	1.3 [b]
----	similar to hippocampus abundant gene transcript 1; tetracycline transporter-like protein (loc295398), mrna	1.0 [a]	1.3 [b]

SUPPLEMENTARY TABLE 2. Supplemental list of genes differentially affected by AOM treatment in distal colonic epithelium of male F344 rats. *(continued)* [1]

Gene symbol	Common	Saline-Injected	AOM-Injected
IX. EST, function unclassified			
----	similar to galactose-1-phosphate uridylyltransferase (gal-1-p uridylyltransferase) (udp-glucose--hexose-1-phosphate uridylyltransferase) (loc298003), mrna	1.0 [a]	1.3 [b]
----	transcribed sequences	1.0 [a]	1.3 [b]
----	similar to ubiquinol-cytochrome c reductase complex core protein 2, mitochondrial precursor (complex iii subunit ii) (loc293448), mrna	1.0 [a]	1.3 [b]
----	similar to protein translocation complex beta; protein transport protein sec61 beta subunit (loc298068)	1.0 [a]	1.3 [b]
----	similar to unr-interacting protein (serine-threonine kinase receptor-associated protein) (loc297699)	1.0 [a]	1.3 [b]
----	similar to germinal histone h4 gene (loc364721)	1.0 [a]	1.3 [b]
----	l81136cds ratrps2r1a rattus norvegicus (strain r21) rps2r1 preliminary dna, complete cds	1.0 [a]	1.3 [b]
----	transcribed sequences	1.0 [a]	1.3 [b]
----	transcribed sequences	1.0 [a]	1.3 [b]
----	similar to discs large homolog 7 (loc289997), mrna	1.0 [b]	1.2 [b]
----	transcribed sequence with weak similarity to protein ref:np_035569.1 (m.musculus) sry-box containing gene 13 [mus musculus]	1.0 [b]	0.7 [a]
----	dd6a4-1 mrna, partial sequence	1.0 [b]	0.7 [a]
----	line retrotransposable element 3	1.0 [b]	0.7 [a]
----	transcribed sequences	1.0 [b]	0.7 [a]
----	x53581cds#5 rnlined r.norvegicus long interspersed repetitive dna containing 7 orf's	1.0 [b]	0.7 [a]
----	similar to riken cdna 0610038l10 gene (loc317214), mrna	1.0 [b]	0.7 [a]

SUPPLEMENTARY TABLE 2. Supplemental list of genes differentially affected by AOM treatment in distal colonic epithelium of male F344 rats. *(continued)* [1]

Gene symbol	Common	Saline-Injected	AOM-Injected
IX. EST, function unclassified			
----	rc_aa859562 ui-r-e0-bv-b-03-0-ui.s1 rattus norvegicus cdna, 3' end /clone=ui-r-e0-bv-b-03-0-ui /clone_end=3' /gb=aa859562 /gi=2949082 /ug=rn.268 /len=123	1.0 [b]	0.7 [a]
----	m13101cds ratlin4a rat long interspersed repetitive dna sequence line4 (l1rn)	1.0 [b]	0.7 [a]
----	rat mixed-tissue library rattus norvegicus cdna clone rx00687 3', mrna sequence [rattus norvegicus]	1.0 [b]	0.7 [a]
----	rc_aa894148 est197951 rattus norvegicus cdna, 3' end /clone=rspar57 /clone_end=3' /gb=aa894148 /gi=3021027 /ug=rn.15739 /len=448	1.0 [b]	0.6 [a]
----	similar to leucine-rich repeat-containing 8 (loc311846), mrna	1.0 [b]	0.6 [a]

[1] Data expressed as mean-fold change differences standardized to the SHAM (saline-injected) animals (n=12/group). Superscripts denote significant main effects for carcinogen treatment ($P < 0.05$).

SUPPLEMENTARY TABLE 3. Supplemental list of genes differentially expressed by dietary treatment in distal colonic epithelium of male F344 rats. [1]

Gene symbol	Gene Title	AIN	BB	SF
I. Cell cycle, cell growth and maintenance, apoptosis				
Pmp22	peripheral myelin protein 22	1.0 [a]	1.4 [b]	1.0 [a]
Btg1	b-cell translocation gene 1	1.0 [a]	1.3 [b]	1.0 [a]
S100a10	s-100 related protein, clone 42c	1.0 [a]	1.2 [b]	1.0 [a]
Ccnd2	cyclin d2	1.0 [a]	1.2 [b]	0.9 [a]
Bok	bcl-2-related ovarian killer protein	1.0 [a]	1.1 [a]	1.4 [b]
Btg3	b-cell translocation gene 3	1.0 [a]	1.0 [a]	1.4 [b]
Pcna	proliferating cell nuclear antigen	1.0 [ab]	0.8 [a]	1.1 [b]
Rpa2	p32-subunit of replication protein a	1.0 [ab]	0.8 [a]	1.1 [b]
Cdc25b	cell division cycle 25b	1.0 [b]	0.8 [a]	1.0 [b]
II. Channel, transporters, & carriers				
Fxyd4	fxyd domain-containing ion transport regulator 4	1.0 [a]	1.4 [b]	1.1 [a]
Slc15a1	solute carrier family 15 (oligopeptide transporter), member 1	1.0 [a]	1.4 [b]	0.9 [a]
LOC64201	2-oxoglutarate carrier	1.0 [a]	1.2 [ab]	1.3 [b]
Calb1	calbindin 1	1.0 [ab]	1.1 [b]	0.7 [a]
Abcb6	atp-binding cassette, sub-family b (mdr/tap), member 6	1.0 [a]	1.0 [ab]	1.3 [b]
Slc25a10	solute carrier family 25 (mitochondrial carrier; dicarboxylate transporter), member 10	1.0 [a]	1.0 [a]	1.3 [b]
Abcb1a	atp-binding cassette, sub-family b (mdr/tap), member 1a	1.0 [a]	1.0 [a]	2.0 [b]
Apob	apolipoprotein b	1.0 [a]	1.0 [a]	1.3 [b]
Abcc3	atp-binding cassette, sub-family c (cftr/mrp), member 3	1.0 [a]	1.0 [a]	1.2 [b]
III. Electron transport, oxidoreductase, detoxification				
Cat	catalase	1.0 [a]	1.0 [a]	1.4 [b]
---	metallothionein	1.0 [a]	0.9 [a]	3.5 [b]
Gstp2	glutathione s-transferase, pi 2	1.0 [ab]	0.8 [a]	1.1 [b]

SUPPLEMENTARY TABLE 3. Supplemental list of genes differentially expressed by dietary treatment in distal colonic epithelium of male F344 rats. ***(continued)*** [1]

Gene symbol	Gene Title	AIN	BB	SF
IV. Energy metabolism				
Bach	brain acyl-coa hydrolase	1.0 [a]	1.1 [ab]	1.5 [b]
Decr1	2,4-dienoyl coa reductase 1, mitochondrial	1.0 [a]	1.1 [a]	1.3 [b]
Cpt1a	carnitine palmitoyltransferase 1, liver	1.0 [a]	1.1 [a]	1.3 [b]
---	farensyl diphosphate synthase	1.0 [a]	1.0 [a]	1.3 [b]
Crot	carnitine o-octanoyltransferase	1.0 [ab]	0.9 [a]	1.3 [b]
Cth	ctl target antigen	1.0 [ab]	0.9 [a]	1.2 [b]
Fbp2	fructose bisphosphatase 2	1.0 [ab]	0.8 [a]	1.1 [b]
V. Enzymes, other				
---	gtp cyclohydrolase 1	1.0 [a]	1.4 [b]	0.9 [a]
---	glutathione s-transferase, mu type 3 (yb3)	1.0 [a]	1.4 [b]	1.1 [ab]
Alas1	aminolevulinic acid synthase 1	1.0 [a]	1.3 [b]	1.1 [a]
LOC81816	ubiquitin conjugating enzyme	1.0 [a]	1.3 [b]	1.2 [ab]
---	aldolase a	1.0 [a]	1.2 [ab]	1.6 [b]
Ggcx	gamma-glutamyl carboxylase	1.0 [a]	1.2 [ab]	1.4 [b]
Gcs1	glucosidase 1	1.0 [a]	1.0 [a]	1.3 [b]
Prpsap1	phosphoribosylpyrophosphate synthetase-associated protein (39 kda)	1.0 [ab]	0.9 [a]	1.1 [b]
Ddost	dolichyl-di-phosphooligosaccharide-protein glycotransferase	1.0 [ab]	0.9 [a]	1.2 [b]
Psat1	phosphoserine aminotransferase 1	1.0 [b]	0.8 [a]	1.0 [b]
---	hypoxanthine guanine phosphoribosyl transferase	1.0 [b]	0.8 [a]	1.0 [b]
Nme2	nucleoside diphosphate kinase	1.0 [b]	0.7 [a]	0.9 [ab]
Qdpr	quinoid dihydropteridine reductase	1.0 [b]	0.7 [a]	0.9 [b]
H2afx	transcribed sequences	1.0 [b]	0.7 [a]	1.0 [b]
Lck	lymphocyte protein tyrosine kinase	1.0 [b]	0.7 [a]	0.9 [ab]
Ddx46	rna helicase	1.0 [b]	0.7 [a]	1.0 [b]
Fen1	flap structure-specific endonuclease 1	1.0 [b]	0.7 [a]	1.0 [b]

SUPPLEMENTARY TABLE 3. Supplemental list of genes differentially expressed by dietary treatment in distal colonic epithelium of male F344 rats. ***(continued)*** [1]

Gene symbol	Gene Title	AIN	BB	SF
VI. Extracellular matrix, cell adhesion, cytoskeleton				
Timp1	tissue inhibitor of metalloproteinase 1	1.0 [a]	1.1 [b]	0.9 [a]
Sparc	secreted acidic cysteine rich glycoprotein	1.0 [b]	1.0 [b]	0.5 [a]
Csrp1	cysteine and glycine-rich protein 1	1.0 [b]	0.8 [a]	1.2 [c]
Myo1b	myosin ib	1.0 [b]	0.7 [a]	0.9 [b]
VII. Immune, defense, inflammation, stress				
Hla-dmb	rt1 class ii, locus dmb	1.0 [a]	1.9 [b]	1.1 [ab]
Hla-dma	rt1 class ii, locus dma	1.0 [a]	1.5 [b]	1.1 [a]
VIII. Nucleic acid binding, transcription regulation				
Ptbp1	polypyrimidine tract binding protein	1.0 [a]	1.1 [ab]	1.3 [b]
Hes1	hairy and enhancer of split 1 (drosophila)	1.0 [a]	1.1 [a]	1.3 [b]
Nfyb	nuclear transcription factor-y beta	1.0 [ab]	0.9 [a]	1.1 [b]
Safb	scaffold attachment factor b	1.0 [ab]	0.9 [a]	1.2 [b]
Nfia	nuclear factor i/a	1.0 [ab]	0.9 [a]	1.3 [b]
H1f0	h1 histone family, member 0	1.0 [b]	0.9 [a]	1.1 [c]
Id3	inhibitor of dna binding 3, dominant negative helix-loop-helix protein	1.0 [ab]	0.9 [a]	1.2 [b]
IX. Signal transduction				
Mir16	membrane interacting protein of RGS16	1.0 [a]	1.3 [b]	1.0 [a]
Stk39	serine threonine kinase 39 (ste20/sps1 homolog, yeast)	1.0 [a]	1.1 [ab]	1.3 [b]
Plcb4	phospholipase c, beta 4	1.0 [a]	0.9 [a]	1.2 [b]
Pdgfra	platelet derived growth factor receptor, alpha polypeptide	1.0 [b]	0.9 [ab]	0.7 [a]
Egf	epidermal growth factor	1.0 [b]	0.7 [a]	1.0 [b]
Fgfr4	fibroblast growth factor receptor 4	1.0 [b]	0.7 [a]	1.0 [b]
Pde2a	phosphodiesterase 2a, cgmp-stimulated	1.0 [b]	0.6 [a]	0.9 [b]

SUPPLEMENTARY TABLE 3. Supplemental list of genes differentially expressed by dietary treatment in distal colonic epithelium of male F344 rats. ***(continued)*** [1]

Gene symbol	Gene Title	AIN	BB	SF
X. Other				
Sqstm1	sequestosome 1	1.0 [a]	1.4 [b]	1.2 [ab]
Myd116	myeloid differentiation primary response gene 116	1.0 [a]	1.4 [b]	1.2 [ab]
Hpcal1	neural visinin-like ca2+-binding protein type 3	1.0 [a]	1.4 [b]	1.3 [b]
Muc3	mucin 3	1.0 [a]	1.4 [b]	1.1 [ab]
---	adipose differentiation-related protein	1.0 [a]	1.3 [c]	1.2 [b]
Lgals8	lectin, galactose binding, soluble 8	1.0 [a]	1.3 [b]	1.1 [ab]
Aplp2	amyloid beta (a4) precursor-like protein 2	1.0 [a]	1.3 [b]	1.3 [b]
Ubqln1	ubiquilin 1	1.0 [a]	1.3 [b]	1.1 [ab]
Unc119	unc-119 homolog (c. elegans)	1.0 [a]	1.3 [b]	1.1 [a]
---	line retrotransposable element 3	1.0 [b]	1.2 [b]	0.6 [a]
Canx	calnexin	1.0 [a]	1.1 [ab]	1.3 [b]
Ftl1	ferritin light chain 1	1.0 [a]	1.1 [b]	0.9 [a]
Utrn	utrophin	1.0 [a]	1.1 [a]	1.6 [b]
---	annexin 1	1.0 [b]	1.0 [b]	0.7 [a]
Mlh1	mismatch repair protein	1.0 [a]	0.9 [a]	1.2 [b]
Apbb3	amyloid beta (a4) precursor protein-binding, family b, member 3	1.0 [a]	0.9 [a]	1.4 [b]
---	heat shock protein precursor	1.0 [ab]	0.9 [a]	1.2 [b]
Pex6	peroxisomal biogenesis factor 6	1.0 [ab]	0.8 [a]	1.1 [b]
Fkbp1a	fk506-binding protein 1a	1.0 [b]	0.7 [a]	0.9 [b]
hr	hairless	1.0 [b]	0.7 [a]	0.9 [ab]
XI. EST, function unclassified				
---	transcribed sequence with weak similarity to protein sp:p04280 (h.sapiens) prp1_human salivary proline-rich protein precursor	1.0 [a]	1.7 [b]	1.7 [b]

SUPPLEMENTARY TABLE 3. Supplemental list of genes differentially expressed by dietary treatment in distal colonic epithelium of male F344 rats. *(continued)* [1]

Gene symbol	Gene Title	AIN	BB	SF
XI. EST, function unclassified				
---	rat mixed-tissue library rattus norvegicus cdna clone rx04144 3', mrna sequence [rattus norvegicus]	1.0 [a]	1.6 [b]	1.8 [b]
---	similar to riken cdna 2310046k01 (loc311536), mrna	1.0 [a]	1.6 [b]	1.2 [ab]
---	similar to riken cdna 0610006f02 (loc366792), mrna	1.0 [a]	1.6 [b]	1.6 [b]
---	transcribed sequences	1.0 [a]	1.4 [b]	1.2 [a]
---	parturition-related protein prp3	1.0 [a]	1.4 [b]	0.9 [a]
---	similar to nima-related protein kinase (loc299204), mrna	1.0 [a]	1.4 [b]	1.4 [b]
---	similar to smooth muscle myosin phosphatase regulatory subunit homolog family member, maternal effect lethal mel-11 (110.6 kd) (mel-11) (loc366002), mrna	1.0 [a]	1.4 [b]	1.2 [ab]
---	transcribed sequences	1.0 [a]	1.4 [ab]	1.6 [b]
---	similar to hypothetical protein flj10579 (loc311328), mrna	1.0 [a]	1.3 [b]	1.1 [a]
---	similar to mkiaa1737 protein (loc314330), mrna	1.0 [a]	1.3 [b]	1.1 [a]
---	transcribed sequences	1.0 [a]	1.3 [b]	1.0 [a]
---	transcribed sequence with weak similarity to protein sp:p00722 (e. coli) bgal_ecoli beta-galactosidase	1.0 [a]	1.3 [b]	1.1 [ab]
---	tumor rejection antigen gp96	1.0 [a]	1.3 [b]	1.5 [b]
---	p55	1.0 [a]	1.3 [b]	1.1 [ab]
---	similar to riken cdna 2210008a03 gene (loc314438), mrna	1.0 [a]	1.2 [ab]	1.3 [b]
---	similar to endoplasmin precursor (endoplasmic reticulum protein 99) (94 kda glucose-regulated protein) (grp94) (erp99) (polymorphic tumor rejection antigen 1) (tumor rejection antigen gp96) (loc362862), mrna	1.0 [a]	1.2 [ab]	1.5 [b]

SUPPLEMENTARY TABLE 3. Supplemental list of genes differentially expressed by dietary treatment in distal colonic epithelium of male F344 rats. *(continued)* [1]

Gene symbol	Gene Title	AIN	BB	SF
XI. EST, function unclassified				
---	transcribed sequence with moderate similarity to protein sp:p00722 (e. coli) bgal_ecoli beta-galactosidase	1.0 [a]	1.1 [a]	1.4 [b]
---	similar to hypothetical protein flj21827 (loc300675), mrna	1.0 [a]	1.1 [ab]	1.3 [b]
---	u11071 rnpabpr2 rattus norvegicus sprague-dawley polyadenylate-binding protein-related protein mrna, 3' end	1.0 [b]	1.0 [b]	0.8 [a]
---	similar to sorting nexin 4 (loc360725), mrna	1.0 [a]	1.0 [a]	1.3 [b]
---	similar to chromosome 10 open reading frame 4; similar to putative acid phosphatase f26c11.1 (loc365458)	1.0 [b]	0.9 [ab]	0.7 [a]
---	transcribed sequences	1.0 [ab]	0.9 [a]	1.1 [b]
---	transcribed sequences	1.0 [b]	0.8 [a]	1.0 [b]
---	rc_aa800849 est190346 rattus norvegicus cdna	1.0 [b]	0.8 [ab]	0.7 [a]
---	transcribed sequences	1.0 [b]	0.8 [a]	1.0 [b]
---	transcribed sequence with moderate similarity to protein pir:a41109 (h.sapiens) a41109 protein-tyrosine-phosphatase	1.0 [b]	0.8 [a]	0.7 [a]
---	similar to n-terminal aceyltransferase 1 (loc310399), mrna	1.0 [b]	0.8 [a]	1.1 [b]
---	transcribed sequence with strong similarity to protein sp:p00722 (e. coli) bgal_ecoli beta-galactosidase	1.0 [b]	0.8 [a]	1.0 [b]
---	similar to acetyl coa transferase-like (loc308100), mrna	1.0 [b]	0.7 [a]	1.0 [b]
---	similar to hypothetical protein flj10241 (loc361520), mrna	1.0 [b]	0.7 [a]	0.7 [a]
---	similar to hcv ns3-transactivated protein 1 (loc299507), mrna	1.0 [b]	0.7 [a]	0.7 [a]
---	similar to transforming protein bmi1 - mouse (loc307151)	1.0 [b]	0.7 [a]	0.8 [a]

SUPPLEMENTARY TABLE 3. Supplemental list of genes differentially expressed by dietary treatment in distal colonic epithelium of male F344 rats. ***(continued)*** [1]

Gene symbol	Gene Title	AIN	BB	SF
XI. EST, function unclassified				
---	transcribed sequences	1.0 [b]	0.7 [a]	0.8 [a]
---	rc_aa892526 est196329 rattus norvegicus cdna	1.0 [b]	0.7 [a]	0.8 [ab]
Smc1l1	smc-like 1 (yeast)	1.0 [c]	0.7 [a]	0.8 [b]
---	similar to nedd4 ww binding protein 4 (loc311676)	1.0 [b]	0.7 [a]	0.7 [a]
---	x05472cds#2 rnrep24r rat 2.4 kb repeat dna right terminal region	1.0 [b]	0.7 [b]	0.3 [a]
---	transcribed sequences	1.0 [b]	0.7 [a]	0.8 [ab]
---	transcribed sequences	1.0 [b]	0.7 [a]	0.8 [ab]
---	erythrocyte protein band 4.1-like 3	1.0 [b]	0.7 [a]	1.1 [b]
---	similar to dual-specificity tyrosine-phosphorylation regulated kinase 4 (loc312721), mrna	1.0 [b]	0.7 [a]	0.9 [b]
---	rc_aa866345 ui-r-a0-bm-a-04-0-ui.s1 rattus norvegicus cdna	1.0 [b]	0.7 [a]	0.9 [b]
---	similar to capg protein (loc297339), mrna	1.0 [b]	0.6 [a]	0.8 [ab]
---	loc363015 (loc363015), mrna	1.0 [b]	0.6 [a]	1.0 [b]
---	similar to protein tyrosine phosphatase 20 (loc301333)	1.0 [c]	0.6 [a]	0.8 [b]
---	similar to hypothetical protein flj20531 (loc303164)	1.0 [b]	0.6 [a]	0.9 [b]
---	similar to smc4 protein (loc295107), mrna	1.0 [b]	0.6 [a]	0.8 [a]
---	similar to expressed sequence au040575 (loc288003)	1.0 [b]	0.6 [a]	1.1 [b]
---	hypothetical loc295337 (loc295337), mrna	1.0 [b]	0.6 [a]	0.7 [a]
---	similar to rac gtpase-activating protein (loc315298), mrna	1.0 [b]	0.5 [a]	0.8 [b]
---	rc_aa860039 ui-r-e0-bz-f-06-0-ui.s2 rattus norvegicus cdna	1.0 [b]	0.5 [a]	0.7 [b]

SUPPLEMENTARY TABLE 3. Supplemental list of genes differentially expressed by dietary treatment in distal colonic epithelium of male F344 rats. ***(continued)*** [1]

Gene symbol	Gene Title	AIN	BB	SF
XI. EST, function unclassified				
---	transcribed sequence with weak similarity to protein ref:np_071357.1 (h.sapiens) hypothetical protein flj22794; kiaa1895 protein [homo sapiens]	1.0 [b]	0.4 [a]	0.9 [b]
---	transcribed sequences	1.0 [b]	0.4 [a]	0.5 [a]
---	x62951mrna rnpbus19 r.norvegicus mrna (pbus19) with repetitive elements	1.0 [b]	0.4 [a]	0.5 [a]

[1] Data expressed as mean-fold change differences standardized to the AIN group (n=8/group). Superscripts denote significant main effects for diet ($P < 0.05$).