DIETARY FISH OIL AND BUTYRATE INCREASE APOPTOSIS AND DECREASE ABERRANT CRYPT FOCI IN COLON CANCER BY ENHANCING HISTONE ACETYLATION AND P21$^{WAF1/CIP1}$ EXPRESSION

A Thesis

by

KRISTY LYNN COVERT

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2005

Major: Nutrition

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ABSTRACT

Dietary Fish Oil and Butyrate Increase Apoptosis and Decrease

Aberrant Crypt Foci in Colon Cancer by Enhancing Histone Acetylation

and $p21^{Waf1/Cip1}$ Expression. (May 2005)

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We have previously shown that dietary fish oil and fiber, particularly the highly-fermentable pectin, are protective against colon cancer in a rat model of carcinogenesis. Therefore, based upon the current body of literature and our previous experimental findings, we hypothesized that one mechanism by which dietary fish oil+pectin suppress the promotion stage of colon cancer is through butyrate, the fermentation product of fiber, targeting (in particular) the $p21^{Waf1/Cip1}$ gene and, via targeted histone hyperacetylation, inducing its expression. We found that dietary butyrate supplementation increased the concentration of fecal butyrate (mole %) in the distal colon, and that this increase corresponded to an increase in histone H4 acetylation. Similarly, diets supplemented with butyrate increased $p21^{Waf1/Cip1}$ expression despite azoxymethane (AOM) treatment, which was not seen in non-butyrate supplemented diets. Furthermore, fish oil+butyrate diets resulted in the highest levels of apoptosis and the lowest levels of ACF, while corn oil+butyrate diets resulted in the lowest levels of apoptosis and the highest levels of ACF. Thus, it appears that the protective effect of

fish oil+butyrate is due to the unique properties of fish oil, providing an environment in which butyrate's enhancement of histone acetylation and p21 expression are pro-apoptotic, thereby diminishing pre-neoplastic ACF development.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Colorectal cancer incidence

Among the developed nations of the world, cancer is the second-greatest cause of death (WHO, 2005). According to the World Health Organization, 20 million individuals are cancer victims today; within 20 y this number is projected to reach 30 million (WHO, 2005). Specifically, colorectal cancer is the fourth most common cancer worldwide, for both men and women, and it (along with lung, breast, and prostate cancer) increases in incidence proportional to a nation's status of economic development (WHO, 2005).

Within the United States, colorectal cancer is the second leading cause of cancer-related deaths (Ries *et al*, 2004). The National Cancer Institute reports that the risk of developing colon or rectal cancer in a man's lifetime is 1 in 16, while for a woman it is 1 in 17 (Ries *et al*, 2004). In the year 2004, the American Cancer Society predicts that approximately 146,940 individuals will be diagnosed with colorectal cancer, and that 56,730 individuals will die from the disease in 2004 alone (ACS, 2004). Such alarming statistics illustrate the great need for continued research to understand the precise mechanism(s) of colon carcinogenesis, as well as to illuminate promising nutritional approaches geared toward prevention.

This thesis follows the style of the *British Journal of Cancer*.

Combating cancer via nutrition is a logical strategy, as beneficial dietary habits are estimated to prevent from up to 80% of colorectal cancers, while poor dietary choices account for approximately 30% of all cancers in Westernized nations—making diet second only to tobacco as a preventable cause of cancer (Cummings and Bingham, 1998; WHO, 2005). Both epidemiological and mechanistic studies have identified various factors associated with an increased risk of developing colorectal cancer, such as age, a personal or family history of colorectal cancer or adenomatous colorectal polyps, a personal history of chronic inflammatory bowel disease, or a genetic predisposition due to an underlying hereditary polyposis or nonpolyposis syndrome (Markowitz and Winawer, 1999). Such risk factors are, at this time, beyond the scope of human prevention. However, dietary choices strongly influence the incidence of cancer, including that of the colon, and are well-within our capacity to modulate (Steinmetz and Potter, 1996; Wargovich, 1997).

Colorectal carcinogenesis

A great body of evidence suggests that carcinogenesis is a multi-step progression in which normal, healthy cells acquire various abnormal capabilities in their evolution toward cancer (Hanahan and Weinberg, 2000). Most of these abnormal abilities can be attributed to mutations in cell cycle regulators, signal transduction pathways, and/or modulators of apoptosis, the programmed death of the cell. Mutations can generally be divided into two categories: dominant gain-of-function mutations that activate oncogenes, and recessive loss-of-function mutations that silence tumor suppressor genes

(Hanahan and Weinberg, 2000). In the context of a healthy cell, proto-oncogenes and tumor suppressor genes coordinately regulate complex, interdependent signaling pathways. If the normal function of either such protein becomes compromised, evasion of apoptosis and uncontrolled proliferation can occur, leading to an imbalance in the cell birth/death homeostasis and spatial organization within the colonic crypt (Hall *et al*, 1994). Evidence suggests, however, that the suppression of apoptosis is actually more critical in the development of colon tumorigenesis than is an increase of cellular proliferation (Chang *et al*, 1998).

It is not completely clear where such deleterious mutations originate. Generally, cancers are thought to originate from mutations in stem cells, the juvenile, undifferentiated cells with limitless proliferative capacity, and that such mutations are successively passed to daughter cells (Garcia *et al*, 1999). However, recent evidence suggests that, within the colon, neoplastic cells manifest at the top of the crypt (Shih *et al*, 2001), the cellular region considered to be differentiated and senescent (Augenlicht *et al*, 1999b). In a histopathological examination of colorectal adenomas, Shih found that such neoplastic cells possessed abnormal proliferation patterns consistent with advanced cancers, and that they were genetically distinct from those normal cells at the crypt base (Shih *et al*, 2001). Both scenarios, however, clearly validate the critical role of apoptosis in the removal of abnormal cells and the prevention of colorectal cancer. Interestingly, specific dietary factors (i.e., dietary fish oil+pectin fiber) have been shown to increase apoptosis, both in the upper and lower regions of the colonic crypt, and this diet-specific

apoptotic enhancement coincides with lower numbers of colon tumor development (Chang *et al*, 1997).

Lipids

Certain research indicates that it may be the specific type of lipid that is more critical to colon cancer development than the total fat intake (Reddy, 1992). In particular, two classes of polyunsaturated fatty acids (PUFAs) have come under scientific scrutiny as possessing opposing biological properties: omega-3 PUFAs, found in cold-water marine fish, and omega-6 PUFAs, abundant in corn oil and the predominant lipid of Western diets. Fish oil, and specifically omega-3 PUFAs eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), have shown chemoprotective benefits (Roynette *et al*, 2004; Sauer *et al*, 2000). Corn oil, however, and its primary omega-6 PUFA linoleic acid (18:2 n-6) have demonstrated tumor-promoting activities (Sauer *et al*, 1997; Whelan and McEntee, 2004). Furthermore, rats consuming a Western-style diet for an extended period (2 y) developed gross colonic lesions, even without chemical carcinogen treatment (Risio *et al*, 1996). Interestingly, Davidson *et al* recently showed that the benefit of fish oil is in the addition of omega-3 PUFAs to the diet, rather than in a reduction of n-6 PUFAs (Davidson *et al*, 2004). However, the precise mechanisms mediating these divergent actions have not been completely elucidated.

We have previously demonstrated that omega-3 PUFAs decrease cell proliferation, increase apoptosis, decrease oxidative DNA damage, and decrease the incidence of colon adenocarcinomas, as compared to omega-6 PUFAs (Bancroft *et al*,

2003; Chang *et al*, 1997, 1998; Hong *et al*, 2000; Lee *et al*, 1993). Omega-3 PUFAs incorporate into cellular membranes where they alter the membrane's dynamic structure, thereby influencing various signaling complexes. Within the colonocyte plasma membrane for example, omega-3 PUFAs alter the lipid environment of colonic caveolae, highly specialized membrane microdomains important in cell signaling, thereby affecting the localization of certain proteins to the caveolae (Ma *et al*, 2004). For example, dietary fish oil reduces the amount of H-ras and eNOS that anchor to the plasma membrane (Ma *et al*, 2004). This alteration of membrane-bound proteins at a critical signaling domain could be one mechanism by which fish oil beneficially modulates downstream cellular events. Omega-3 PUFAs also incorporate into the mitochondrial membrane. Evidence suggests that dietary consumption of fish oil results in mitochondrial membrane enrichment with omega-3 PUFAs, which is thought to increase the unsaturation of the membrane (Chapkin *et al*, 2002). This increased unsaturation enhances the cell's apoptotic response to carcinogen by making the mitochondria more sensitive to the pro-apoptotic activity of reactive oxygen species (ROS) (Hong *et al*, 2002).

As well as their role in cellular membrane dynamics, fish oil and omega-3 PUFAs appear protective against carcinogenesis via other modalities. Chronic inflammatory conditions are thought to be risk factors for colon cancer (Itzkowitz and Yio, 2004), and omega-3 PUFAs are thought to positively influence such conditions. Omega-3 and omega-6 PUFAs compete for metabolic conversion by cyclooxygenase (COX) and lipoxygenase (LOX) enzymes, which utilize these precursors to produce

various prostacyclins, thromboxanes, leukotrienes, and lipoxins, collectively known as eicosanoids (Roynette *et al*, 2004). Evidence suggests that eicosanoids derived from EPA (an omega-3 PUFA) are less pro-inflammatory than are the eicosanoids from arachidonic acid (an omega-6 PUFA). In fact, such arachidonic acid-derived eicosanoids as PGE_2 are considered to be tumor promoters (Sheng *et al*, 1998). Moreover, fish oil consumption results in decreased arachidonic acid and PGE_2 levels in the colonic mucosa, compared to corn oil (Lee *et al*, 1993). This might be explained by the ability of fish oil (*in vitro* and *in vivo*) to decrease the activity of the COX-2 enzyme, which converts arachidonic acid to prostaglandins and other metabolites and is known to be highly over-expressed in colonic tumors (Singh *et al*, 1997). Thus, fish oil, both by decreasing the availability of arachidonic acid and by decreasing the activity of the COX-2 enzyme, appears to diminish the production of pro-inflammatory, tumor-enhancing eicosanoids.

Fiber

Fiber—any non-digestible carbohydrate or lignin found intrinsic and intact in plants—passes through the mammalian gastrointestinal tract undigested until it reaches the large intestine (Institute of Medicine, 2002; Lupton, 2004). Here, fiber encounters the colon's anaerobic microbial flora, which has the enzymatic capacity to cleave the molecular bonds of fiber. This process is known as microbial fermentation, and results in the secondary production of hydrogen, carbon dioxide, methane, and short chain fatty acids

(SCFAs), of which butyrate is thought to possess unique physiological and chemoprotective properties (Lupton, 2004).

Although many studies detailing colon and rectal cancer epidemiology regard fiber as one homogeneous class, the specific compounds comprising this group are, functionally, quite diverse. With regard to the colon, the degree of a fiber's fermentability should be a primary basis for distinction between fiber sources. More-appropriately, therefore, "fiber" should be sub-divided into highly-fermentable and poorly-fermentable fibers, both of which possess properties that could be chemoprotective. Highly-fermentable fiber, such as pectin and some hemicellulose (found primarily in fruits and vegetables) is readily fermented by the bacterial microflora, yielding a higher content of butyrate and other SCFAs than poorly-fermentable fiber, such as cellulose and wheat bran (found primarily in cereals) (Zoran *et al*, 1997a; 1997b). Poorly-fermentable fibers, however, have been shown to dilute the fecal contents and to speed the transit of feces through the colon, both of which potentially diminish the colonocyte's exposure to carcinogens (Burkitt *et al*, 1972; Gazzaniga and Lupton, 1987). Thus, striking differences exist between the intralumenal activities of these two fiber classes.

Butyrate

Among SCFAs, butyrate has been most extensively examined in colon carcinogenesis. The four-carbon butyrate molecule is the preferred energy source for colonocytes, and is a major SCFA constituent of the colonic lumenal contents (Cummings, 1984; Roediger,

1980). Following fiber fermentation, butyrate enters the colonic epithelial cell via apical membrane proteins known as monocarboxylate transporters (MCT1), which are most abundant in colonocytes lining the surface of the colon (Lambert *et al*, 2002). Interestingly, as cells progress from healthy to malignant, the colonocyte shifts its expression from the MCT1 transporter to the high-affinity glucose transporter (GLUC 1), not normally present in the colon, thus facilitating enhanced glucose and decreased butyrate utilization (Lambert *et al*, 2002). This demonstrates a shift in the metabolic preferences of the malignant colonocyte and illustrates the critical importance of butyrate to a healthy colon.

Certain epidemiological studies have reported that increasing fiber intake negatively correlates with colon and rectal cancer development (Bingham *et al*, 2003; Burkitt, 1969; Howe *et al*, 1992). Other studies, however, indicate that increasing fiber confers no protective benefit (Fuchs *et al*, 1999; McCullough *et al*, 2003; Steinmetz *et al*, 1994). Butyrate has been involved in a similar controversy (Lupton, 2004). For example, *in vitro*, butyrate clearly stimulates various colonic tumor cell lines to undergo cell cycle arrest, differentiation, apoptosis, and to decrease proliferation (Archer *et al*, 1998b; Aukema *et al*, 1997; Hague *et al*, 1993; Schwartz *et al*, 1998). However, butyrate's activities *in vivo* have been inconclusive, similar to the apparent contradictions observed in studies examining dietary fiber (Caderni *et al*, 2001; Hong *et al*, 2002). Although these disparate findings appear difficult to reconcile, some recent evidence suggests that it is the particular class of dietary lipids interacting with a distinctive type of fiber (or butyrate) that may determine whether or not fiber and

butyrate protect against colorectal cancer. While Hong demonstrated that *ex vivo* butyrate treatment increased apoptosis in colonocytes of rats fed fish oil (Hong *et al*, 2002), Caderni reported that *in vivo* butyrate supplementation neither increased the level of apoptosis nor the expression of $p21^{Waf1/Cip1}$, a protein implicated in cell-cycle arrest and apoptosis (Caderni *et al*, 2001). However, Caderni's butyrate supplementation was in addition to a diet in which corn oil was the lipid source.

In recent preliminary investigations, we have reported that the anti-carcinogenic activity of fish oil may be enhanced by the presence of butyrate, and that butyrate may exacerbate the pro-carcinogenic effects of corn oil (Covert *et al*, 2003). To date, however, the mechanisms behind this observation have not been elucidated, nor have effects of *in vivo* butyrate administration, provided by dietary supplementation and targeted to the colon, in conjunction with both fish oil- and corn oil-enriched diets, been reported. Therefore, it is by this approach that the current study could help define the circumstances in which treatment with a highly-fermentable fiber and/or butyrate is efficacious.

Histones

Within the nucleus, DNA is packaged into tight coils known as nucleosomes, which are considered to be the basic units of eukaryotic chromatin (Kornberg, 1974). Each nucleosome consists of 146 base pairs of DNA wrapped around a core of 8 individual histone proteins, known as a histone octomer (Marks *et al*, 2001). There are two groups of highly-conserved DNA-binding histones: eight "core" histone proteins (two each of

protein H3, H4, H2A, and H2B) and one non-core "linker" histone protein (H1) (Grunstein, 1997). These two types of histones work together to hold the DNA into nucleosome units and to fold the repeating units into repressed higher-order chromatin fibers (Strahl and Allis, 2000). Each histone protein is comprised of a globular "body" domain and a positively-charged amino-terminal "tail" domain (Strahl and Allis, 2000). DNA is held tightly wrapped around the histone octomer by the attraction between negatively-charged phosphate groups on the surface of the DNA helix and positively-charged amino acid residues on the histone amino-terminal tails. In this condensed configuration, genetic material is not available for access by the transcriptional machinery—it is kept silent.

Histone acetylation

Butyrate influences many processes within the cell, including various DNA-template activities such as DNA replication, repair, and gene transcription (Akey and Luger, 2003; Bird *et al*, 2002; Kemp *et al*, 2005; Strahl and Allis, 2000). It appears that one means by which butyrate modulates such activities is, in part, by altering the status of nucleosomal histone acetylation. The reversible, dynamic process of histone acetylation is the best understood histone modification, to date (Grunstein, 1997). There appears to be a high degree of clinical and pathological significance to histone acetylation, as Ono *et al* found that decreasing acetylation of histone H4 was correlated with increasing gastric cancer progression, as well as increased depth of tumor invasion and metastasis (Ono *et al*, 2002).

Histone tail regions are composed of various amino acids protruding from the histone body; they provide an accessible surface for contact by other proteins and a convenient site for many post-translational modifications, including acetylation (Bradbury, 1992; Strahl and Allis, 2000). Lysine residues are the most common targets of acetylation, which induces the conformational changes in chromatin structure necessary for specific DNA sequences to be accessed by transcription factors (Strahl and Allis, 2000). This particular modification is fundamental to transcriptional regulation (Kornberg and Lorch, 1999). In its silent, condensed state, DNA is wrapped around histones whose lysine residues are hypoacetylated. However, when acetyl groups are added to the lysine residues, the positive charge of the lysines is neutralized, weakening the bond between the histone and the DNA; the chromatin "relaxes," allowing the DNA to unwind and make a particular region of the genome accessible for transcription (Marks *et al*, 2001; Strahl and Allis, 2000). Two classes of enzymes work in concert with one another to facilitate the steady-state acetylation or deacetylation of histones. Histone acetyltransferases (HATs) transfer acetyl groups from acetyl coenzyme A to the lysine residues, facilitating active gene transcription; histone deacetylases (HDACs) cleave acetyl groups from the histone tail regions, keeping genes silenced (Kornberg and Lorch, 1999). To achieve active gene expression or active gene repression, multi-protein complexes recruit HATs or HDACs to specific regulatory sequences within the genome, including loci for transcription factors (Glass and Rosenfeld, 2000).

It is well established that butyrate induces transient histone hyperacetylation, and that it does so by acting as a noncompetitive, reversible inhibitor of the histone

deacetylase (HDAC) enzyme, both *in vitro* and *in vivo* (Boffa *et al*, 1992; Candido *et al*, 1978; Sealy and Chalkley, 1978). While butyrate suppresses the HDAC enzyme, it does not affect the enzymatic activity of HAT; thus, butyrate shifts the balance of acetylation to favor a hyperacetylated status, consistent with active transcription, by preventing the removal of acetyl groups and allowing them to accumulate (Vidali *et al*, 1978). In general, butyrate's targeted HDAC-inhibition leads to the upregulation of particular sequences within the genome, as evidenced by the well-documented induction of the $p21^{Waf1/Cip1}$ gene by butyrate (Archer *et al*, 1998a).

Apoptosis

Programmed cellular suicide, or apoptosis, is one important method of colonocyte death. Butyrate has been shown to play a critical role in inducing apoptosis, both *in vitro* and *in vivo* (Augenlicht *et al*, 1999a; Caderni *et al*, 1998; McBain *et al*, 1997). For example, in transgenic mice unable to effectively metabolize butyrate, colonic apoptosis was decreased by more than 90% (Augenlicht *et al*, 1999a). Fish oil has also been shown to increase apoptosis in the colon (Hong *et al*, 2002, 2003), and the combination of n-3 PUFAs and fiber (or butyrate) significantly enhances the level of apoptosis in the colon over that of fish oil alone (Chang *et al*, 1997, 1998). Amplification of apoptosis may be another mechanism by which n-3 PUFA and fiber/butyrate co-treatment is protective.

Various events precipitate apoptosis, leading to the removal of damaged, abnormal, or aging cells from the colon. Colon cells are "born," or originate, at the bottom of the crypt and begin migrating up the crypt axis until they reach the lumenal

surface (Lipkin, 1989). In the lower two-thirds of the crypt column, cells experience active proliferation; as they continue to move upward, they become progressively more differentiated, primarily into goblet cells, losing their replicative capacity until they reach senescence at the crypt surface (Augenlicht *et al*, 1999b). Apoptosis can occur at any point along the crypt axis once the cell has left the proliferative zone; however, if the normal aged cell has not already undergone apoptosis by the time that it reaches the crypt surface, it will be shed from the crypt through a form of apoptosis known as anoikis, which occurs when the cell no longer has contact with the extracellular matrix (Augenlicht *et al*, 1999b; Shanmugathasan and Jothy, 2000). Thus, apoptosis plays a vital role in maintaining the homeostatic balance between cell birth and cell death within the crypt (Hall *et al*, 1994). Various studies have identified a reduction in apoptosis to be correlated with an increased risk of colon cancer (Bedi *et al*, 1995; Chang *et al*, 1998; Tomlinson and Bodmer, 1995). Likewise, apoptosis becomes progressively more inhibited as colorectal cancer progresses (Bedi *et al*, 1995).

Two distinct pathways can execute apoptosis: the intrinsic apoptotic pathway or the extrinsic apoptotic pathway (Riedl and Shi, 2004). Although each reacts to different stimuli and responds with different signaling cascades, a common protease—cysteine aspartyl-specific proteases, termed “caspases”—functions as the primary executioner for both pathways. Two types of caspases drive apoptosis: initiator caspases and effector caspases (Riedl and Shi, 2004; Shanmugathasan and Jothy, 2000). Initiator caspases are auto-activated, while effector caspases are activated when they are cleaved by initiator

caspases (Hajra and Liu, 2004; Riedl and Shi, 2004). The cleavage of caspases instigates a chain of irreversible activity, ultimately culminating in the cell's death.

The intrinsic, or mitochondrial, pathway is initiated by stimuli such as overexpression of oncogenes or irreparable damage to the DNA. The intrinsic apoptotic pathway is mediated through the leakage of cytochrome-c, a mitochondrial protein, in response to mitochondrial damage (Hajra and Liu, 2004; Riedl and Shi, 2004). Once in the cytoplasm, cytochrome-c binds with various proteins and leads to the establishment of the apoptosome, a multi-protein complex that facilitates the auto-activation of initiator caspase-9. Activated caspase-9 then cleaves downstream effector caspases, resulting in their proteolytic activation and subsequent death activities (Hajra and Liu, 2004; Riedl and Shi, 2004). In contrast to the intrinsic pathway, the extrinsic, or receptor-mediated, pathway is initiated in response to the binding of specific ligands to death receptors (e.g., Fas, TRAIL) on the cell surface (Hajra and Liu, 2004; Riedl and Shi, 2004). Upon ligand binding, the adapter molecule FADD is recruited to the cell surface. FADD then recruits many initiator caspases to the area; the initiator caspases self-activate and then activate the effector caspases in the same manner as that of the intrinsic pathway (Hajra and Liu, 2004; Riedl and Shi, 2004).

There are several general phases of apoptosis. The first phase commences after the cell receives the initiating stimulus, thus provoking an apoptotic response (Vaux and Strasser, 1996). Such stimuli include, but are not limited to, loss of contact with the extracellular matrix, exposure to specific toxins, UV or ionizing radiation, ligand binding to death receptors, DNA damage or errors in DNA replication, and the activity

of reactive oxygen species (ROS) (Shanmugathasan and Jothy, 2000; Vaux and Strasser, 1996). The second phase involves the detection and transduction of the death signal. The apoptotic message is relayed via signal transduction pathways to the death effector machinery—caspases—waiting in the cytoplasm. Here, they are in the inactive, pro-caspase form, as caspases do not need to be newly synthesized upon receipt of an apoptotic signal (Vaux and Strasser, 1996). The third phase of apoptosis is the effector phase, chiefly accomplished by the activated caspases. At this time, the classical apoptotic morphology begins to emerge, characterized by cell shrinkage, mitochondrial membrane blebbing, nuclear degradation, and DNA fragmentation (Shanmugathasan and Jothy, 2000). As the DNA fragments, the remaining double-strand breaks can be identified by gel electrophoresis, producing a specific pattern that appears as a 180 base pair "ladder," a classic indicator of apoptosis (Vaux and Strasser, 1996). DNA strand-breaks are also the characteristic apoptotic feature identified in the TUNEL bioassay for apoptosis. Finally, during the postmortem period, the nuclear chromatin condenses and DNA is degraded. Cellular remnants are packaged into apoptotic bodies, which are then phagocytized or sloughed into the passing lumenal contents (Vaux and Strasser, 1996).

p21$^{\text{Waf1/Cip1}}$

One of the earliest events in the butyrate-mediated apoptotic pathway, in vitro, is the induction of the cyclin-dependent kinase inhibitor (CDKI), p21$^{\text{Waf1/Cip1}}$ (p21) (Augenlicht *et al*, 1999b). p21 is a 164 amino acid protein within a family of potent cyclin dependant kinase (CDK) inhibitors (Harper *et al*, 1993). p21 can be

transcriptionally activated through various means. As the "genome guardian" protein, p53 induces p21 expression in response to DNA damage in order to temporarily arrest the cell cycle and allow time for DNA repairs to be made prior to replication (el-Deiry *et al*, 1993; Waldman *et al*, 1995); however, p21 can also be induced independently of p53, such as with butyrate treatment (Archer *et al*, 1998a; Michieli *et al*, 1994).

Specific proteins are responsible for regulating cell cycle progression. CDKs associate with cyclins, and together they drive the cell cycle (Gartel and Tyner, 1999). At low levels, p21 constitutively contributes to this function by serving as an assembly factor for CDK/cyclin complexes, acting as a central molecule in the four-member complex of active CDKs, which includes one CDK, one cyclin, one proliferating cell nuclear antigen (PCNA), and one p21 protein (Zhang *et al*, 1993, 1994). However, elevated levels of p21 can result in more than one p21 molecule per CDK-quaternary complex, thus inhibiting CDKs and leading to G1-phase and (potentially) G2-phase arrest (Dulic *et al*, 1998; Harper *et al*, 1995). Therefore, the determination for whether or not p21 promotes or inhibits cell cycle progression appears to be contingent upon the concentration of p21 present.

In order to block cell cycle progression most effectively, a second p21 molecule associates with the CDK/cyclin complex at one (or both) of its two cyclin binding regions and at its CDK binding region (Chen *et al*, 1996). This binding prevents the CDK/cyclin complex from phosphorylating other proteins (e.g., pRb) necessary for cell cycle progression (Harper *et al*, 1993). p21 can also block cyclical progression by competing with positive cell cycle regulators for the binding sites on CDK/cyclin

complexes, and by competing with the CDK/cyclin substrates; p21 is even capable of disrupting CDK/cyclin/substrate complexes after they are formed (Dotto, 2000; Shiyanov *et al*, 1996). Additionally, p21 is capable of directly preventing DNA replication by binding to the proliferating cell nuclear antigen (PCNA) subunit of DNA polymerase (Luo *et al*, 1995). Therefore, p21's chemoprotective activities may originate from several avenues, including the ability to halt cell cycle progression, promote differentiation, and prevent DNA replication—all of which are potentially protective.

The exact mechanism by which butyrate treatment induces the expression of $p21^{Waf1/Cip1}$ is not completely understood. The p21 promoter region contains six GC-rich sequences coding for binding sites specific to a family of zinc-finger transcription factors known as Sp1 factors (Gartel and Tyner, 1999). The GC-rich-, Sp1-binding sites can be employed to induce p21 in response to various stimuli; for example, butyrate and other HDAC inhibitors, such as trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA), utilize the GC-rich sequences to mediate their upregulation of p21 (Huang *et al*, 2000; Nakano *et al*, 1997; Sowa *et al*, 1997). In the case of butyrate-mediated p21 induction, Sp1 and Sp3 (two Sp1-family transcription factors) bind to the Sp1-binding sites within p21's promoter and are thought to regulate butyrate's activity (Nakano *et al*, 1997). Recent evidence suggests that, in a cooperative regulatory fashion, Sp1 serves as a negative transcription regulator via binding to HDAC1, while Sp3 acts as a positive transcription regulator via association with the HAT protein, p300 (Doetzlhofer *et al*, 1999; Kishikawa *et al*, 2002; Sowa *et al*, 1999). Thus, it is hypothesized that, upon administration of an HDAC-inhibitor (such as butyrate), the inhibition of histone

deacetylation will be sufficient to overcome the negative activity of HDAC1 (associated with Sp1), to such an extent that the HAT activity of p300 (associated with Sp3) can induce the conformational change in chromatin necessary for p21 to be transcribed (Huang *et al*, 2000; Merchant *et al*, 2003).

Like butyrate, the specific mechanisms and circumstances governing p21 activity are also not clearly understood. Most *in vitro* evidence demonstrates that p21 is anti-apoptotic (Gorospe *et al*, 1997; Wang and Walsh, 1996), while *in vivo* studies generally find p21 to be pro-apoptotic (Yang *et al*, 2001). Nonetheless, various studies demonstrate that p21 plays a critical role in mediating the death cascade. Duttaroy *et al* demonstrated that, *in vitro*, 3T3 fibroblasts required an upregulation of p21 above the basal level for apoptosis to occur, and that even partial suppression of p21 approximately doubled cell survival (Duttaroy *et al*, 1997). *In vivo*, Yang *et al* (2001), employing a transgenic mouse model with inactivated p21, reported that the loss of p21 resulted in several deleterious consequences. First, $p21^{-/-}$-mice experienced increased formation of Apc-initiated tumors, which were correlated with decreased apoptosis, decreased differentiation, and increased proliferation; second, the loss of p21 exacerbated the Western-style (corn oil-rich) diet, resulting in enhanced tumorigenesis; and third, such animals had significantly decreased lifespans (Yang *et al*, 2001).

The conditions under which p21 functions in a pro- or anti-apoptotic manner are not completely clear. One hypothesis is that to facilitate apoptosis, p21 must first be cleaved, such as by butyrate-activated caspases, either to (a) inactivate anti-apoptotic p21, possibly by altering its subcellular localization, or to (b) liberate a pro-apoptotic

cleavage product of p21 (Chai *et al*, 2000a). In one study using the A549 human lung carcinoma cell line, caspase-3 cleaved p21, resulting in the loss of p21's C-terminal domain and nuclear localization sequence, and generated a 15 kilodalton (kD) fragment. This cleavage instigated the carcinoma cells' transition from a state of growth arrest to one of active apoptosis (Zhang *et al*, 1999). The same progression of caspase-3 activation, p21 cleavage, and the appearance of a 15 kD fragment has also been demonstrated in epithelial cancer cell lines, including colon cell lines, in response to both butyrate-induced apoptosis and zinc depletion-induced apoptosis (Chai *et al*, 2000a, 2000b). Thus, the cleavage of p21 by caspase-3 may be one key step in the apoptotic cascade following butyrate treatment.

Aberrant crypt foci

Long before the colorectal tumor stage, single colonic crypts experience aberrant morphological changes, thought to be key cytological features of a pre-neoplastic state (Bird, 1995). Such putative preneoplastic colonic lesions are known as aberrant crypt foci (ACF). ACF are characterized as having irregularly shaped, dilated lumenal crypt openings, being larger in size (width and/or height) than neighboring normal crypts, possessing a thickened epithelial lining around the crypt, and experiencing increased proliferative activity causing them to extend upward into the lumen (Bird, 1995). ACF are generally monoclonal, meaning that one progenitor cell is selected and initiated, followed by clonal expansion of cells identical to the original progenitor (Bird, 1995). The proliferating cell colony then becomes the preneoplastic lesion. ACF are also

dynamic lesions that may undergo phases of remodeling or regression during their development, although the reasons for this are unknown (Bird and Good, 2000).

ACF were first described by Bird in 1987, and were discovered only in those colons subjected to carcinogen treatment (Bird, 1987). Established colon cancer promoters, such as corn oil and beef tallow, enhance ACF, whereas known inhibitors, such as fish oil, decrease ACF; this behavior parallels that of colon tumors (Bird, 1995). Proliferative atypia, consistently seen in colorectal cancer, is also characteristic of ACF. Abnormal proliferation in aberrant crypts is evidenced by an unusual number of AGNOR (argyrophilic nucleolar organization region) bodies found in the lesions. AGNOR bodies represent loops of ribosomal DNA in interphase cells, indicative of cellular activity and protein synthesis; increasing numbers of AGNOR bodies are associated with neoplasia (Bird, 1995). Interestingly, ACF nuclei were found to possess both larger AGNOR bodies and higher numbers of AGNOR bodies than their normal neighboring crypts, again suggesting that ACF are lesions progressing toward neoplasia (Bird, 1995). Similarly, ACF were shown to possess abnormal levels of proliferation, as detected by BrdU incorporation, which mirrored the proliferative activity of tumors within the same animals (Pretlow *et al*, 1994).

ACF demonstrate other characteristics that parallel tumorigenesis, beyond that of proliferative atypia. ACF have been shown to possess mutations in the *K-ras* gene, a mutation consistently documented in both colon adenomas and tumors (Shivapurkar *et al*, 1994; Vivona *et al*, 1993). ACF also appear to acquire enhanced resistance to apoptosis, as they increase in multiplicity (Magnuson *et al*, 1994). Consistent with this,

Pretlow demonstrated that rats injected with AOM developed both HM ACF, those lesions with 4 or more abnormal crypts per focus, and tumors, and that those rats who developed tumors also had significantly more HM ACF than rats without tumors (Pretlow *et al*, 1992). In humans, ACF have also been documented both in patients at high risk for developing colorectal cancer and those already diagnosed with colorectal cancer; however, ACF were greater in those patients with colorectal tumors than without, indicating that ACF are a high risk factor for colon cancer in humans (Pretlow *et al*, 1991; Takayama *et al*, 1998). Therefore, their well-documented role in both human and animal-model carcinogenesis warrants the use of ACF as prognostic indicators of colorectal cancer development.

Although some evidence challenges their validity as preeminent tumor biomarkers (Yamada *et al*, 2000, 2001), ACF are generally regarded as predictive of colon cancer (Magnuson *et al*, 1994). Specifically, HM ACF appear to be the more-sensitive indicators of colon tumorigenesis (Pretlow *et al*, 1992). HM ACF represent a more advanced preneoplastic state than do those lesions with fewer (3 or less) abnormal crypts, termed low-multiplicity aberrant crypt foci (LM ACF) (Bird, 1995). While *in vivo* butyrate treatment has been shown to either increase or decrease the development of total ACF and HM ACF depending upon the dietary lipid provided, the specific mechanism(s) of butyrate's role in their development are not clearly known (Covert *et al*, 2003).

Hypothesis and specific aims

Based upon the current body of literature and our previous experimental findings, we hypothesize that one mechanism by which dietary fish oil and pectin suppress the promotion stage of colon carcinogenesis is through butyrate, the fermentation product of fiber, targeting (in particular) the *p21*$^{Waf1/Cip1}$ gene and, by acting as a histone deacetylase inhibitor, inducing its expression. Within the pro-apoptotic environment created by fish oil, this activity contributes to an increase in apoptosis and subsequent decrease in aberrant crypt formation, thereby suppressing tumorigenesis.

To test this hypothesis and to delineate the effects of *in vivo* butyrate supplementation within both fish oil- and corn oil-enriched environments, we sought to:

A. Determine the number and multiplicity level of aberrant crypt foci within the proximal and distal colon,
B. Analyze and measure the short chain fatty acid content of fecal samples at termination,
C. Measure the amount of apoptosis occurring in the distal colon,
D. Quantify the expression and localization of the p21$^{Waf1/Cip1}$ protein in distal colonic crypts, and
E. Assess the degree of histone hyperacetylation occurring in the nuclei of distal colonic crypts.

CHAPTER II

MATERIALS AND METHODS

Azoxymethane-induced rat model of carcinogenesis

The rodent/azoxymethane (AOM)-model of colon carcinogenesis closely mirrors the development of colorectal cancer in humans (Ahnen, 1985). AOM has been shown to induce alterations in many critical genes and signaling molecules, including β-catenin, k-ras, APC (adenomatous polyposis coli), COX-II, cyclin D1, and various cyclin-dependant kinases, all of which are implicated in human tumorigenesis (DuBois *et al*, 1996; Maltzman *et al*, 1997; Takayama *et al*, 1998; Vivona *et al*, 1993; Wang *et al*, 1998). Of special significance to this work, the AOM-induced model of carcinogenesis clearly distinguishes between the initiation and promotion stages of tumorigenesis (Reddy *et al*, 1991). Also, the rodent/AOM-model of colorectal cancer has proven to be valuable in studying apoptosis and its regulation, and has been extensively utilized in our laboratory previously (Chang *et al*, 1998; Hong *et al*, 1999). Precancerous biomarkers such as ACF, used to help detail the developmental progression of colorectal cancer, are difficult to assess in humans. ACF, in particular, are difficult to quantify *in vivo* (Bird and Good, 2000), but are readily exhibited in an AOM-treated rodent model, when the colons are examined *ex vivo* (Covert *et al*, 2003). Such features validate our use of the AOM-induced rodent model to study colon carcinogenesis.

Animals and experimental groups

The experimental procedures of this study were approved by the University Animal Care Committee of Texas A&M University and conformed to those guidelines set forth by the National Institutes of Health. Eighty male weanling Sprague Dawley rats (28 d old, from Harlan Sprague-Dawley, Houston, TX, USA) were utilized in this study. Experimental diet/treatment groups were constructed following a 2 x 2 x 2 factorial design—two dietary lipid sources (corn oil or fish oil), two sodium butyrate pellet supplementation groups (+/- sodium butyrate pellets), and two injection protocols (saline or AOM). Experimental diets were composed of a basal diet mix (Harlan Teklad, Madison, WI, USA), plus the respective experimental lipid source (corn oil or fish oil, Degussa Bioactives, Champaign, IL, USA), with or without the addition of slow-release sodium butyrate pellets (Valpharma S.A., Serravalle, Republic of San Marino, Italy) (Table 1). Each experimental diet was comprised of 85% basal mix and 15% fat, by weight (Table 1). Both fish oil-enriched diet groups (with and without butyrate supplementation) were comprised of 11.5% fish oil by weight and 3.5% corn oil by weight, to prevent essential fatty acid deficiencies. All oils were supplemented with antioxidants. Alpha- and gamma-tocopherol are endogenously present within corn oil (0.7 mg total tocopherol/g corn oil); therefore, to normalize these levels between the lipids, synthetic tocopherol was added to the fish oil in the form of MTS-70 (Archer Daniels Midland, Decatur, IL, USA). Lot number MTS1001-63 of MTS-70 contained 0.718 g tocopherol/g MTS-70, as determined by lot analysis. Therefore, to achieve an amount of total tocopherol equivalent to that present in corn oil, 0.973 g MTS-70 was

Table 1 Experimental diet composition

Ingredient	g / 100 g total diet
Dextrose	51.06
Casein	22.35
Methionine	0.34
Mineral mix, AIN-76	3.91
Vitamin mix, AIN-76	1.12
Choline bitartrate	0.22
Cellulose	6.00
Corn oil[a]	15.00
or Fish oil [a,b] / Corn oil	11.50/3.50

[a]TBHQ and [b]α, γ tocopherols were added as antioxidants. TBHQ (0.25 g/kg total oil) supplied via Tenox 20-A (Gillco, Vista, CA, USA), to prevent oxidative damage to oils. Synthetic α, γ tocopherols (0.698 g/kg fish oil) supplied via MTS-70 (Archer Daniels Midland, Decatur, IL, USA), to normalize antioxidant levels within fish oil to those endogenously present in corn oil.

added per kg fish oil, supplying 0.698 g total tocopherol per kg fish oil. Both corn oil and fish oil were also supplemented with Tenox 20-A (Gillco, Vista, CA, USA), to provide the antioxidant tertiary butylhydroquinone (TBHQ). Only 20% of Tenox 20-A is TBHQ; therefore, as TBHQ should be 0.025% (or 0.25 g/kg) of the total oil to prevent oxidative damage to the lipids, 1.25 g Tenox 20-A per kg oil were supplied to provide sufficient antioxidant protection. Sodium butyrate pellets were added to both corn oil- and fish-oil +butyrate diet groups, at 1.5% of the diet by weight. All diets were stored in –80° C freezers to prevent degradation.

Upon arrival at Texas A&M University's Laboratory Animal Research Residence (LARR), a temperature- and humidity-controlled facility with a daily 12 h light/dark photoperiod, rats were immediately separated and housed in individual polycarbonate cages with raised double grid floors, to help prevent coprophagy and to minimize their consumption of bedding. Each animal was then weighed and randomly assigned to one of eight experimental diet/treatment groups (10 rats per group); each group's starting mean weight was approximately equal (data not shown). The rats were given a 1 wk acclimatization period before starting the experimental diet, during which time they consumed a standard rat diet. Following the period of acclimatization, animals were provided their respective experimental diet (fresh diet was supplied each morning) and water, which they freely consumed for a total of 11 wk. After 3 wk of experimental diet consumption, the rats received their first subcutaneous injection of AOM (Sigma Chemical, St. Louis, MO, USA), a colon-specific carcinogen at a dosage of 15 mg/kg body weight, or an equivalent volume of saline (Sigma Chemical, St. Louis, MO, USA)

as a control, followed by the second injection 1 wk later. Of the total 80 rats, 40 received two injections of AOM, and 40 received two injections of saline.

Termination and tissue sample collection

Seven weeks after the second injection, rats were terminated via CO_2 euthanasia and cervical dislocation within a 4 h timeframe (to diminish circadian variability). The colons were then harvested, and 1 cm was removed from each of the distal and proximal ends and fixed in 4% paraformaldehyde (PFA; Electron Microscopy Sciences, Hatfield, PA, USA) for 4 h to preserve the unique colon morphology, critical for immunohistochemical analysis. Fixed tissues were rinsed in graded alcohol washes before being embedded in paraffin wax for extended storage. The remaining colon was divided longitudinally. Half of the colon was prepared for the aberrant crypt foci (ACF) assay, while the alternate half was scraped and the mucosal contents stored at –80°C for possible RNA and protein analysis.

Aberrant crypt foci assay

One half of the colon was collected and prepared for aberrant crypt foci analysis. Upon removal from the animal, the colon was irrigated with cold, sterile Dulbecco's phosphate-buffered saline (Gibco, Grand Island, NY, USA) and opened. The colonic tissue was placed mucosal-side up on a sheet of Whatman #1 filter paper (Whatman International, Maidstone, England) and fixed in 4% PFA for 24 h. After transfer to 70% ethanol for storage, the tissues were then stained with 0.5% methylene blue (Sigma

Chemical, St. Louis, MO, USA) and examined microscopically. Aberrant crypts were enumerated for each animal, both as the total number of ACF (noting the multiplicity of each) and as the number of high multiplicity ACF, foci with 4 or more aberrant crypts per focus.

Short chain fatty acid quantification

Upon termination, feces from the proximal and distal colon were collected for gas chromatographic analysis of the volatile short chain fatty acids (SCFAs) (Zoran *et al*, 1997b). At the time of termination, fecal samples were separated into labeled cryotubes and immediately frozen in liquid nitrogen, to be stored at –80°C. Frozen fecal samples were ground into a frozen powder, weighed, and prepared for analysis. Two internal standards, 2-ethylbutyric acid (2-EtBA; Aldrich Chemical, Milwaukee, WI, USA) and heptanoic acid (HA; Alltech Associates, Deerfield, IL, USA), were added to each sample. 2-EtBA, a compound belonging to the same chemical class as SCFAs, was added to the sample at the beginning of the extraction process to account for any sample loss due to extraction inefficiency; the second internal standard, HA, was added to the sample once the extraction process was complete, to determine how the GC responded to the sample. Powdered 2-EtBA (0.0014 g) was dissolved in 25 mls of 70% ethanol to achieve a concentration of 0.511 mM; this served as the stock solution of the 2-EtBA internal standard. 1 ml of 2-EtBA stock solution was added to each fecal sample weighing more than 0.2 g; 0.5 ml of 2-EtBA was added to each fecal sample weighing less than 0.2 g. After adding the 2-EtBA, the SCFAs extracted into the ethanol at 4ºC

overnight. Samples were then spun down in the chilled (4ºC) Eppendorf 5717 R centrifuge for 20 min at 15,000 RCF. The supernatant was drawn off and transferred into 2 ml glass vials. Immediately prior to GC analysis, 200 µl of 0.830 mM heptanoic acid internal standard stock solution was added to the sample, along with 20 µl of 10% ortho-phosphoric acid (Fisher Scientific, Fair Lawn, NJ, USA) to protonate the SCFAs and help keep them in solution. A 1 µl aliquot of each sample solution was then injected into the Varian 3900 GC, a single channel, splitless injection, fixed configuration, capillary column gas chromatograph. The GC was equipped with a HP-FFAP capillary column 30 meters long, with an internal capillary diameter of 0.53 mm and 1 µm film coating thickness inside capillary. The injector temperature was 200ºC, causing the samples to vaporize. The oven temperature was held at 60ºC for 2.5 min, and increased 8ºC per min until it reached 185ºC and then remained at this temperature for 10 min. At this point, solutes eluted through the column at a constant column flow of 7.0 ml He/min and were electronically detected. After this, the oven increased 6ºC per min until it reached 200ºC where it held for 10 min, and then continued to increase 50ºC per min until it reached an internal temperature of 220ºC and held for 5 min to burn off any residue inside the oven. The make-up flow of N was 23 ml/min to force the sample across the flame, while the H2 flow was 30 ml/min and the compressed air flow was 300 ml/min to maintain the flame burning. The Varian Star Workstation software generated a chromatogram of the identity and amount of solutes present in each sample aliquot. Six SCFAs (acetic, propionic, butyric, isobutyric, valeric, and isovaleric acids) were detected and quantified.

Apoptosis assay

Apoptosis was assessed *in situ* by the TUNEL assay. This methodology identifies DNA that has been cleaved into 180 base pair particles, a classic characteristic of apoptosis. A cell undergoing apoptosis possesses high numbers of double and/or single stranded DNA fragments, which presents many free 3'-OH termini of DNA within the nucleus, compared to normal or proliferating cells. Utilizing the ApopTag Apoptosis Detection System (Serologicals Corporation, Norcross, GA, USA) free 3'-OH are labeled, stained, and visualized by light microscopy, thereby illuminating specific cells undergoing apoptosis within the histological architecture of the colonic crypt section.

Following harvest and fixation, paraffin-embedded tissues were cut into non-serial 4 μm-thick sections. After deparaffinization in xylene (Fisher Scientific, Fair Lawn, NJ, USA), rehydration in graded alcohols, and washing in PBS, tissues were pre-treated with a nonspecific serine protease enzyme (Proteinase K Solution, 10 μg/ml PBS; Ambion, Austin, TX, USA). Incubation in Proteinase K served to digest the DNA-binding proteins associated with PFA-induced cross-linking fixation, which can prevent DNA fragments from being effectively extracted and processed. To diminish non-specific background staining, the endogenous peroxidase within the tissue was quenched. We saturated the peroxidase with 0.3% hydrogen peroxide (Sigma Chemical, St. Louis, MO, USA) in 100% methanol (Fisher Scientific, Fair Lawn, NJ, USA), which permanently inactivated the enzyme, preventing it from catalyzing the chromogenic substrate 3-3'-diaminobenzidene (DAB; Sigma Chemical, St. Louis, MO, USA).

Tissues were then sufficiently processed to label the free 3'-OH termini via the terminal deoxynucleotidyl transferase (TdT) enzyme.

TdT enzymatically adds nucleotides, some of which are labeled with the digoxigenin antigen, to the free DNA ends. The labeled and unlabeled nucleotides are added randomly, but in a ratio that best facilitates the binding of the anti-digoxigenin antibody to the digoxigenin antigen; this antibody is conjugated to a peroxidase reporter molecule. In the presence of the peroxidase reporter enzyme, subsequent application of the chromagen DAB caused an insoluble brown precipitate to form at the antigen-antibody binding site, thus identifying the highly concentrated areas of DNA strand-breaks as brown bodies within the crypt axis. Positive controls were created by exposing selected tissue sections to DNase I (Ambion, Austin, TX, USA), an enzyme that chemically-induces DNA strand breaks simulating apoptosis. Negative controls were DNase I-incubated sections processed without the TdT enzyme—therefore, no brown precipitate should have developed. The tissues were then counterstained with 0.05% methyl green (Aldrich Chemical, Milwaukee, WI, USA), dehydrated in reverse-graded alcohols, and wet mounted with Permount (Fisher Scientific, Fair Lawn, NJ, USA) before being coverslipped.

Apoptosis was scored by microscopic examination. Positive cells were identified as those possessing both intense brown staining from the TUNEL assay and specific morphological features characteristic of apoptosis (Kerr *et al*, 1995). Fifty well-oriented crypt columns were selected, and both the total number of cells per column and the specific position of positive cells were recorded. Apoptosis was represented as a

percentage, which was calculated by dividing the number of apoptotic cells per column by the total number of cells per column and multiplying by 100, known as the apoptotic index for each crypt column.

p21$^{Waf1/Cip1}$ assay

The cyclin dependent kinase inhibitor p21$^{Waf1/Cip1}$ (p21) was visualized *in situ* via immunohistochemistry following an optimized procedure based on previous work (Ruan *et al*, 1998; Sinicrope *et al*, 1998; Viale *et al*, 1999). Non-serial 4 μm sections were cut from paraffin-embedded tissues. After deparaffinization in xylene, rehydration in graded alcohols, and washing in PBS, the endogenous peroxidase was quenched as described in the apoptosis assay (above). Following peroxidase quenching, tissues were pre-treated by microwave heating with Retrievit Target Retrieval Solution (InnoGenex, San Ramon, CA, USA), a process known as antigen unmasking. This technique breaks the DNA-binding proteins associated with PFA-induced cross-linking fixation, allowing specific proteins of interest to be accessed by antibodies. In order to locate and visualize the p21 protein, tissue sections were incubated at 4ºC overnight with primary purified mouse anti-p21 monoclonal antibody (BD Pharmingen, San Diego, CA, USA), at a 1:10 dilution in 1% normal rabbit serum (Jackson ImmunoResearch, West Grove, PA, USA). Negative control sections were incubated overnight with 1% normal rabbit serum only, rather than primary antibody solution. After overnight saturation of the p21 protein by the anti-p21 monoclonal antibody, tissues were incubated for 2 h with a

biotinylated rabbit anti-mouse secondary antibody (DakoCytomation, Carpinteria, CA, USA), at a 1:250 dilution in 1% normal rabbit serum.

Tissues were then incubated with the Vectastain Elite avidin and biotinylated horseradish peroxidase macromolecular complex (ABC; Vector Laboratories, Burlingame, CA, USA), a peroxide-based reporter enzyme system that binds the biotinylated secondary antibody. The efficacy of this reporter enzyme complex is based on the high natural affinity of avidin, a large glycoprotein, for the vitamin biotin; once bound, the interaction is irreversible. Because the secondary antibody was conjugated to biotin (biotinylated), incubation with the proprietary Vectastain Elite ABC Kit solutions sequentially bound avidin to the biotin of the secondary antibody, which was then bound by the biotinylated horseradish peroxidase enzyme. The peroxidase reporter enzyme interacted with the DAB chromagen as described in the apoptosis assay (above), producing the brown precipitate characteristic of a positive signal. After incubation with DAB, tissues were dehydrated in reverse-graded alcohols and wet mounted with Permount before being coverslipped.

The staining intensity of p21 was assessed by microscopic examination. Images were captured with NIH Image Software 4.0. Each nucleus within the right crypt column of 15 well-oriented crypts for each of 64 rats (eight per treatment group) was circled for semi-quantitative analysis, to determine the mean p21 stain intensity per nucleus. Both the average stain intensity per nucleus and the cell position were recorded. Background staining was detected by determining the average stain intensity of a 25-by-25 pixel square, captured at the lumenal surface of the crypt column as near

to the highest labeled cell as possible. This value was subtracted from the average stain intensity of each nucleus. After subtracting the background intensity, the average stain intensity of all nuclei within each crypt column were added together and divided by the total number of nuclei per crypt column, to determine the mean p21 stain intensity per nucleus per crypt column.

Histone H4 acetylation assay

Acetylated histone H4 was visualized *in situ* via immunohistochemistry following an optimized procedure based on previous work (Mariadason, 2004; Warrell *et al*, 1998). The immunohistochemical procedure for detecting the acetylation status of the histone H4 protein was identical to that of the p21 assay described above, excluding only the specific primary and secondary antibodies and blocking sera used. The primary antibody used to detect acetylated histone H4 was the Anti-acetyl-Histone H4 rabbit polyclonal antibody diluted 1:750 (Upstate, Lake Placid, NY, USA), and the secondary antibody was the Biotin-SP-conjugated AffiniPure Goat Anti-Rabbit antibody diluted 1:200 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Each antibody was diluted in 1% Normal Goat Serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The staining intensity of acetylated histone H4 was assessed by microscopic examination, image acquisition, and image analysis as described above in the p21 assay. Consistent with the p21 assay, acetylated histone H4 staining was assessed semi-quantitatively by circling each nucleus within the right crypt column of 15 well-oriented crypts, for each of 64 rats (eight per treatment group), followed by the

subtraction of background staining, and determination of the average stain intensity per nucleus per crypt column, to assess the mean acetylated histone H4 stain intensity.

Statistical analysis

Body weights and food intake data were analyzed using a two-way ANOVA. Short chain fatty acid quantification, aberrant crypt foci, apoptosis, $p21^{Waf1/Cip1}$, and histone acetylation assays were analyzed by three-way ANOVA, as well as by using a split plot designed mixed model procedure (PROC MIXED) in SAS (Hong, 2000). The relationship between apoptosis and total ACF, as well as p21 and HM ACF, was determined by regression analysis in SAS. Differences between treatments were regarded as significant when P values were <0.05.

CHAPTER III

RESULTS

Changes in body weight, but not in food intake, between diet groups

Three individual 48-h food intake assessments were conducted: pre-first injection, post-second injection, and pre-termination. There were no main effects of diet or injection treatment on food intake between the experimental groups, at any food intake assessment point (data not shown). However, despite the consistency of food intake between groups, differences were found between their overall weight gains. Overall weight gain was determined by subtracting the weights at the beginning of the pre-first injection food intake from the weights at the end of the pre-termination food intake. AOM-injected rats in both corn oil-supplemented groups (with and without butyrate) demonstrated significantly less weight gain than all other treatment groups (except saline-injected corn oil+butyrate rats) (Figure 1). This is consistent with what we have previously demonstrated (Chang *et al*, 1997); however, after statistical analysis with weight as a covariate, we found no effect of weight on any of the outcome measurements.

Concentration of total SCFAs (µmoles/gwwf) and butyrate (mole %) in fecal material

After conducting 48-h fecal collections, we found no differences in the total amount of fecal material (g) excreted between the four diet groups (data not shown). Therefore, all

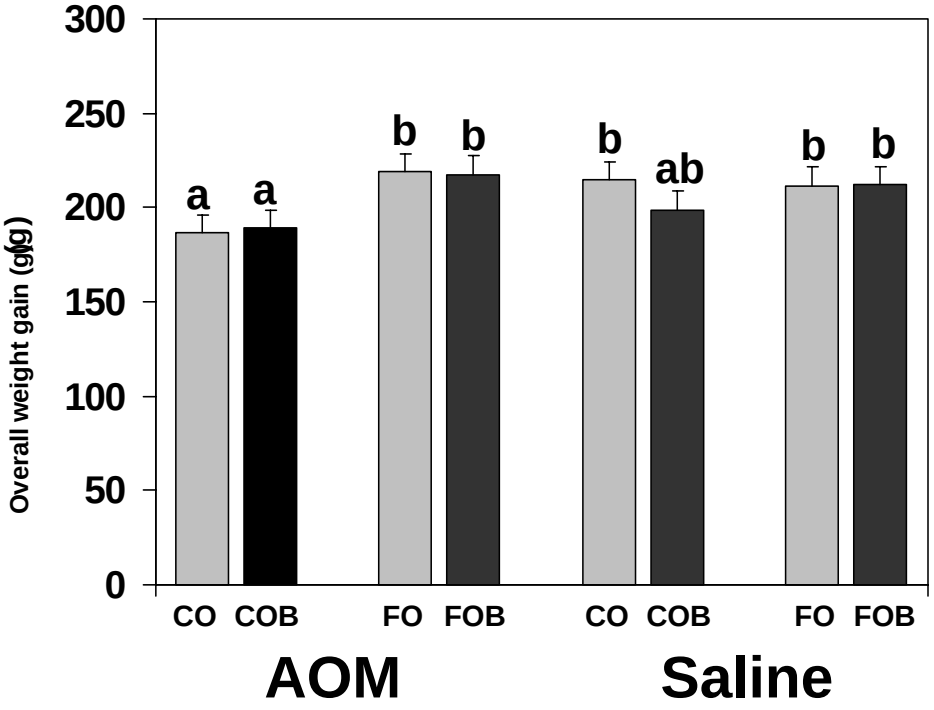

Figure 1 AOM-injected rats fed corn oil diets, with and without butyrate, gained less weight than rats given all other diet/injection treatments. Data are means ± SEM for 80 rats (n=10 rats per diet/treatment group). Bars with different letters are significantly different (P<0.05). CO = corn oil; COB = corn oil+butyrate; FO = fish oil; FOB = fish oil+butyrate; AOM = azoxymethane.

data regarding the short chain fatty acids (SCFAs) present in the feces were reported as concentrations, either as µmoles/gram wet weight feces (µmoles/gwwf) or as a percentage (mole %) of the total SCFAs.

The combined total concentration (µmoles/gwwf) of the six SCFAs that were measured—acetic, propionic, butyric, isobutyric, valeric, and isovaleric acids—was greater in the proximal colon than in the distal colon, and this was observed in the fecal material of both corn oil and fish oil fed animals (corn, $P<0.0001$; fish, $P<0.0001$; Figure 2). This represents a main effect of region on the concentration of total SCFAs. In the distal colon, dietary butyrate supplementation increased the total concentration of SCFAs in the feces (µmoles/gwwf) compared to that of non-butyrate supplemented diets, regardless of lipid ($P=0.0477$; Figure 3). Butyrate supplementation made no difference in the concentration of total SCFAs within feces from the proximal colon ($P=0.8637$; Figure 3).

Within the proximal colon, however, lipid affected the concentration of fecal butyrate (mole %), as fish oil-enriched diets produced higher fecal butyrate levels than corn oil-enriched diets ($P=0.004$; Figure 4). There was no effect of butyrate supplementation ($P=0.3663$) or injection treatment ($P=0.3728$) on proximal fecal butyrate levels. Within the distal colon, regardless of injection treatment, the concentration of fecal butyrate (mole %) was higher in the fish oil+butyrate groups than corn+butyrate groups ($P=0.0422$; Figure 5). However, the effect of fish+butyrate versus corn+butyrate was clearly attributable to the saline animals ($P=0.0176$; Figure 6), as rats

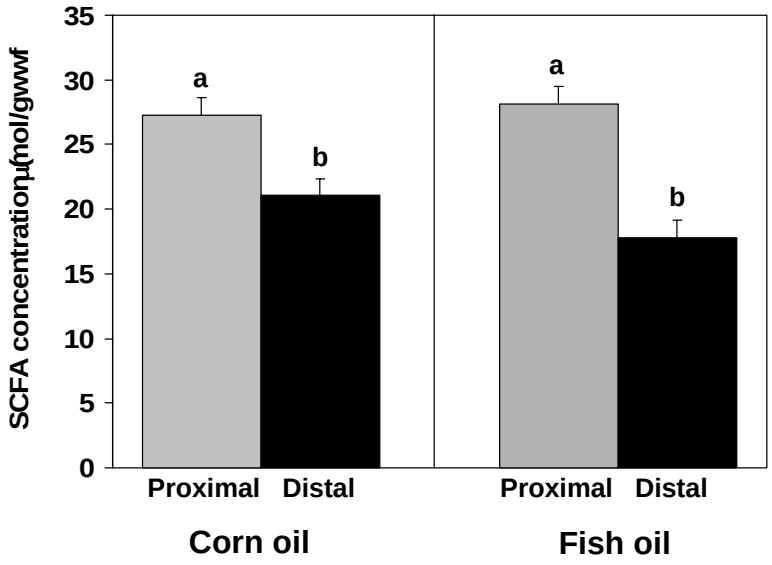

Figure 2 The combined total SCFA concentration was greater in the proximal colon than the distal colon of both corn oil- and fish oil-fed rats, regardless of injection treatment or butyrate supplementation. Data are means ± SEM for 77 rats (n=37 rats per corn oil diet proximal region, and n=40 rats per corn oil diet distal region; n=38 rats per fish oil diet, proximal region, and n=39 rats per fish oil diet, distal region). Bars with different letters are significantly different (P<0.0001). SCFA = short chain fatty acid; µmol/gwwf = micromoles per gram wet weight feces.

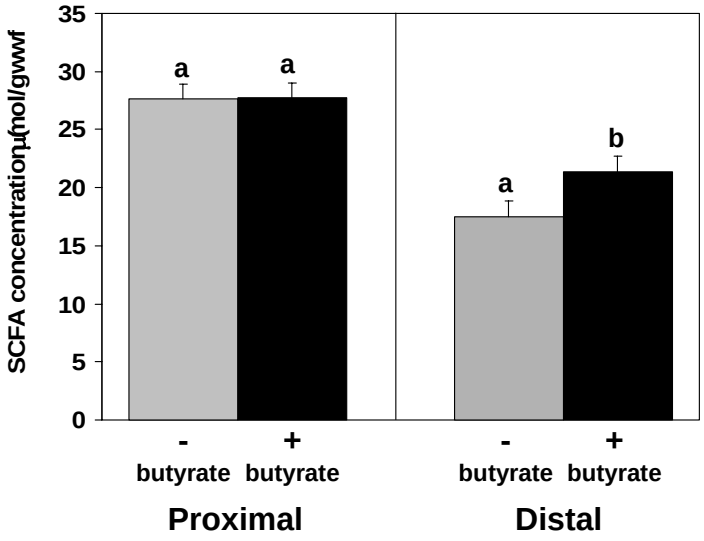

Figure 3 Dietary butyrate supplementation increased the concentration of total SCFAs over that of non-butyrate supplemented diets in the distal colon only. Data are means ± SEM for 77 rats (n=38 rats per proximal region –butyrate group, n=37 rats per proximal region +butyrate group; n=39 rats per proximal region –butyrate group, n=40 rats per proximal region +butyrate group). Bars with different letters are significantly different (P<0.05). SCFA = short chain fatty acid; µmol/gwwf = micromoles per gram wet weight feces.

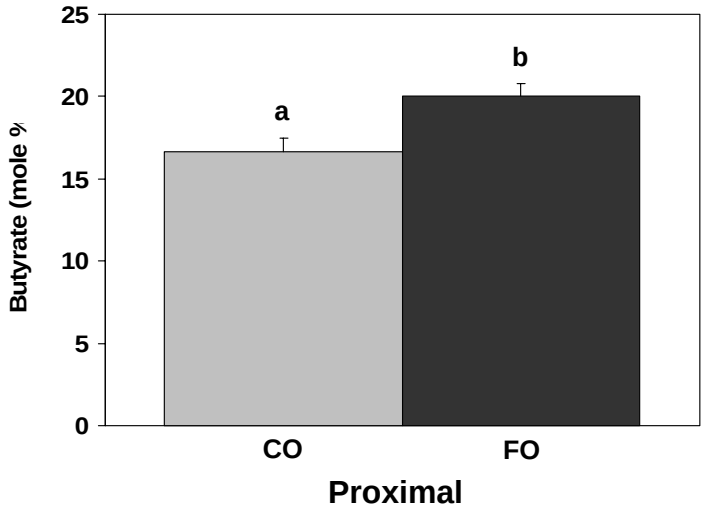

Figure 4 In the proximal colon, fish oil-enriched diets resulted in greater fecal butyrate levels than corn oil-enriched diets. There was no effect of butyrate supplementation or injection treatment (data not shown). Data are means ± SEM for 74 rats (n=36 rats per corn oil group; n=38 rats per fish oil group). Bars with different letters are significantly different (P=0.004). CO = corn oil; FO = fish oil.

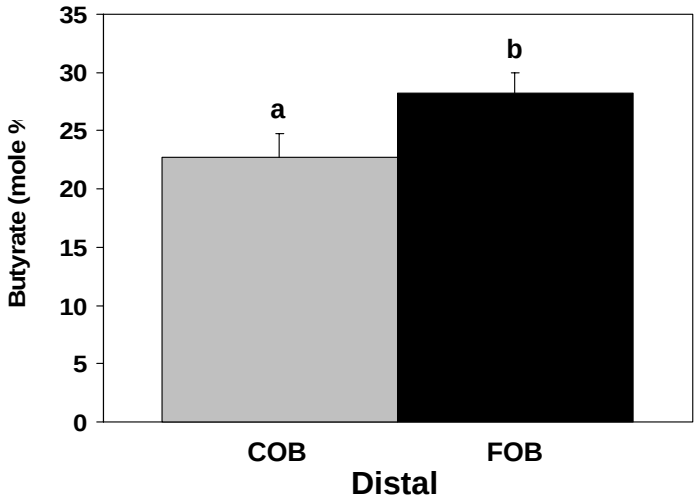

Figure 5 In the distal colon, fish oil+butyrate diets resulted in greater fecal butyrate levels than corn oil+butyrate diets, regardless of injection treatment. Data are means ± SEM for 38 rats (n=19 rats per diet group). Bars with different letters are significantly different (P<0.05). COB = corn oil+butyrate; FOB = fish oil+butyrate.

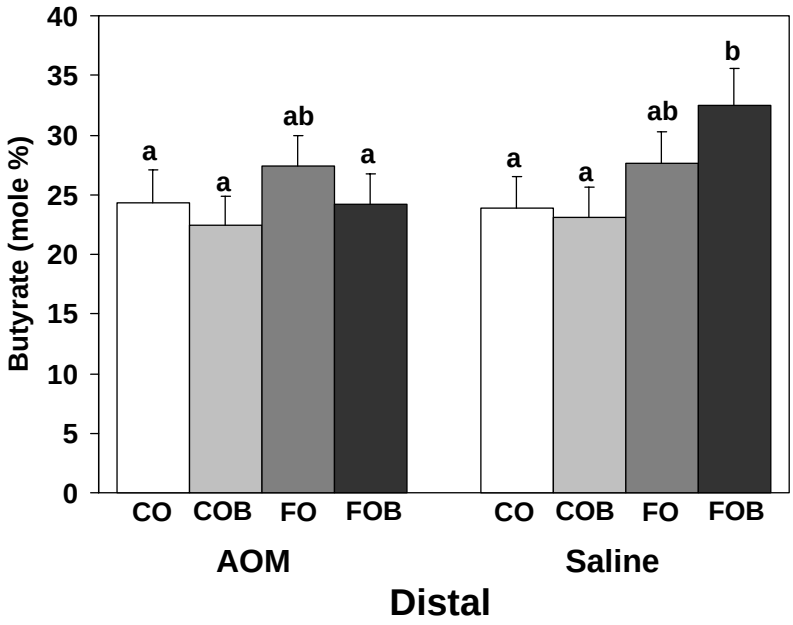

Figure 6 FOB-fed rats had higher levels of fecal butyrate in the distal colon than CO- or COB-fed rats, but only if they were injected with saline. Rats injected with AOM did not have different levels of fecal butyrate, regardless of lipid or butyrate supplementation. FOB-fed rats injected with saline had higher distal fecal butyrate levels than all other groups, except for FO-fed rats injected with AOM or saline. Data are means ± SEM for 78 rats (n=10 rats per diet/treatment group, except n=9 rats per FOB AOM group and n=9 rats per COB Saline group). Bars with different letters are significantly different ($P<0.05$). CO = corn oil; COB = corn oil+butyrate; FO = fish oil; FOB = fish oil+butyrate; AOM = azoxymethane.

injected with AOM did not have higher distal fecal butyrate levels with fish oil+butyrate versus corn oil+butyrate consumption (P=0.6224; Figure 6). Among non-butyrate supplemented rats, there was no difference in the distal fecal butyrate concentration between fish oil- and corn oil-enriched groups, either injected with saline or AOM (Figure 6).

In the distal colon of rats fed corn oil, AOM versus saline treatment made no difference in the concentration of fecal butyrate (mole %), either with or without butyrate supplementation (Figure 6). Similarly, in rats fed fish oil without butyrate supplementation, there was also no difference in the concentration of fecal butyrate with AOM versus saline treatment (Figure 6). However, in rats fed fish oil with butyrate supplementation, the AOM-injected group experienced lower fecal butyrate levels than those injected with saline and fed the same diet (P=0.0378; Figure 6).

Enumeration of total ACF and HM ACF in the colon

Due to the absence of aberrant crypts in rats injected with saline, all aberrant crypt foci results are reported from animals exposed to carcinogen. Regardless of diet, both the number of total aberrant crypt foci (total ACF; P<0.0001; Figure 7) and the number of high-multiplicity aberrant crypt foci (HM ACF; P<0.0001; Figure 8) were higher in the distal colon than the proximal colon. Regardless of colon region, however, there were fewer total ACF in rats fed a fish oil+butyrate diet compared to a corn oil+butyrate diet (P<0.0001, Figure 9).

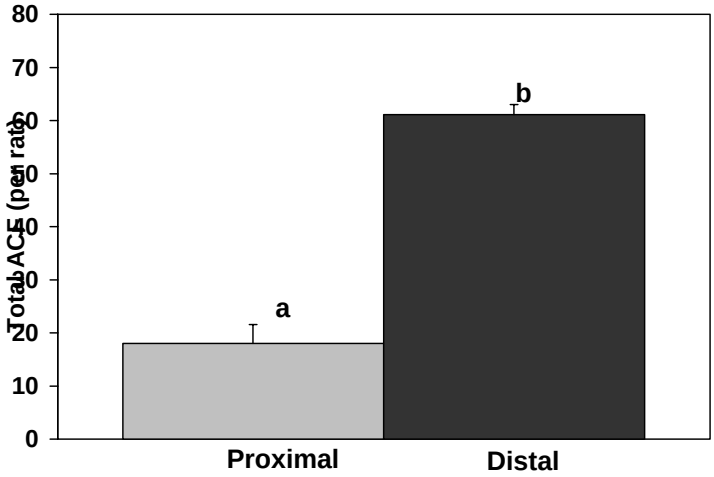

Figure 7 Total ACF were greater in the distal colon than the proximal colon, regardless of diet. All rats were injected with AOM. Data are means ± SEM for 40 rats (n=40 rats per proximal colon region; n=39 rats per distal colon region). Bars with different letters are significantly different (*P*<0.0001). ACF = aberrant crypt foci.

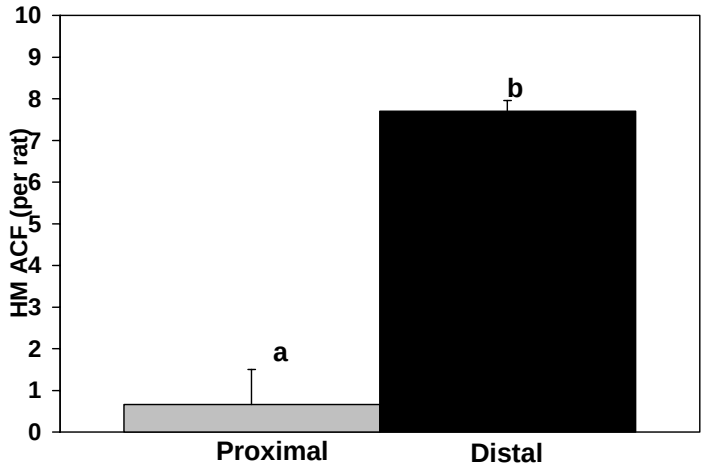

Figure 8 HM ACF were greater in the distal colon than the proximal colon, regardless of diet. All rats were injected with AOM. Data are means ± SEM for 40 rats (n=40 rats per colon region). Bars with different letters are significantly different (*P*<0.0001). HM ACF = high multiplicity aberrant crypt foci.

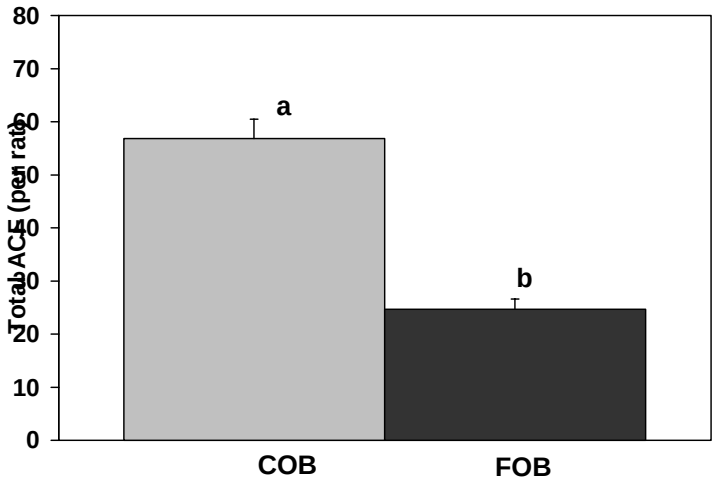

Figure 9 Regardless of colon region, COB-fed rats experienced more total ACF than FOB-fed rats. All rats were injected with AOM. Data are means ± SEM for 40 rats (n=39 colon regions per COB diet group, 20 proximal regions and 19 distal regions; n=40 colon regions per FOB diet group, 20 proximal regions and 20 distal regions). Bars with different letters are significantly different (*P*<0.0001). COB = corn oil+butyrate; FOB = fish oil+butyrate; ACF = aberrant crypt foci.

Moreover, feeding corn oil+butyrate resulted in more total ACF than any other diet group ($P<0.005$; Figure 10), regardless of region. Corn oil+butyrate fed rats also developed the greatest number of HM ACF compared to all other diets ($P<0.011$; Figure 11), while rats consuming fish oil+butyrate developed the fewest number of HM ACF compared to all other diets ($P<0.038$; Figure 11). Specifically, rats consuming fish oil+butyrate diets exhibited 88% fewer HM ACF lesions per colon than rats in the corn oil+butyrate diet group ($P<0.0001$).

Apoptotic index

Because the ACF phenotype was enhanced in the distal colon, and because the effect of diet on ACF formation was similar in both colon regions, all assays investigating apoptosis were conducted in tissue from the distal colon. In saline injected rats, corn+butyrate and fish+butyrate diets did not enhance apoptosis compared to their non-butyrate supplemented counterparts (Figure 12). However, in AOM-injected rats, supplementation of butyrate did enhance apoptosis, but only in those rats consuming fish oil (with fish oil+butyrate, $P=0.014$; Figure 13). Thus, butyrate was unable to promote apoptosis in response to a carcinogen when corn oil was the dietary lipid source. Furthermore, fish+butyrate diets enhanced apoptosis over corn+butyrate diets, regardless of treatment (Figure 12 and 13).

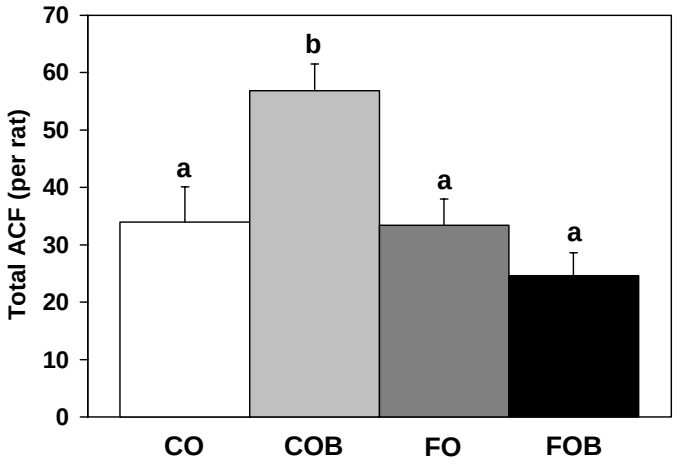

Figure 10 Regardless of colon region, COB diets resulted in more total ACF than any other diet group. All rats were injected with AOM. Data are means ± SEM for 39 rats (n=19 colon regions per COB diet group; n=20 colon regions per CO, FO, and FOB diet groups). Bars with different letters are significantly different (*P*<0.005). COB = corn oil+butyrate; CO = corn oil; FO = fish oil; FOB = fish oil+butyrate; ACF = aberrant crypt foci.

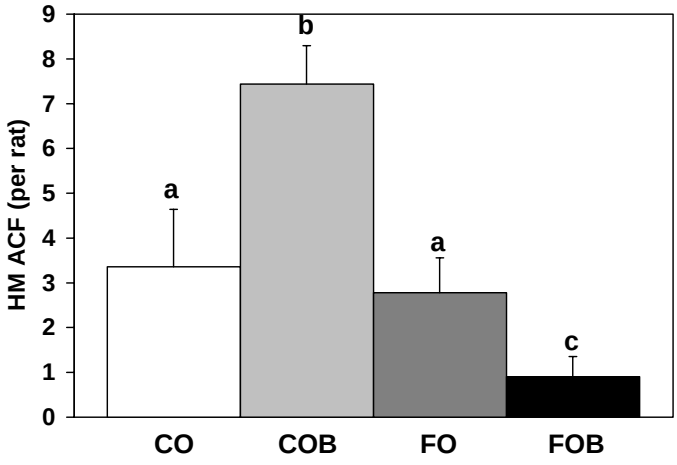

Figure 11 Regardless of colon region, COB diets resulted in more HM ACF while FOB diets resulted in fewer HM ACF than any other diet groups. All rats were injected with AOM. Data are means ± SEM for 40 rats (n=20 colon regions per diet group). Bars with different letters are significantly different (*P*<0.04). COB = corn oil+butyrate; CO = corn oil; FO = fish oil; FOB = fish oil+butyrate; HM ACF = high multiplicity aberrant crypt foci.

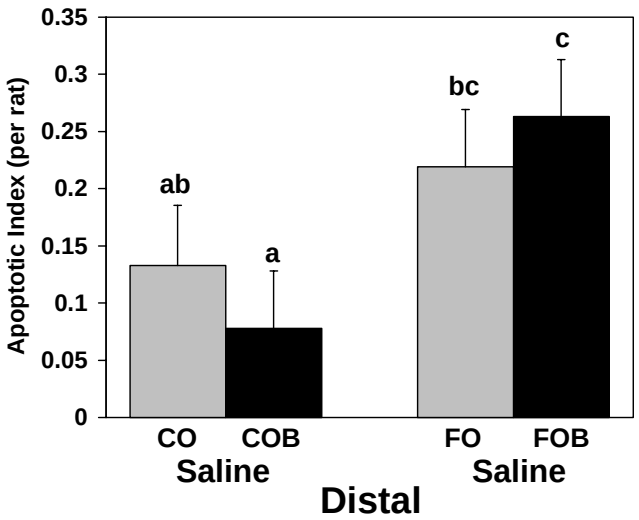

Figure 12 In rats injected with saline, butyrate-supplemented diets did not increase the level of apoptosis compared to non-butyrate supplemented diets. Apoptosis was measured in the distal colon only. Apoptosis is expressed as an apoptotic index (number of apoptotic cells / total number of cells per crypt column, x 100). Data are means ± SEM for 39 rats (n=9 rats per CO Saline group; n=10 rats per COB Saline, FO Saline, and FOB Saline groups; *P*>0.03). For each rat, 50 well-oriented crypt columns were assessed. CO = corn oil; COB = corn oil+butyrate; FO = fish oil; FOB = fish oil+butyrate.

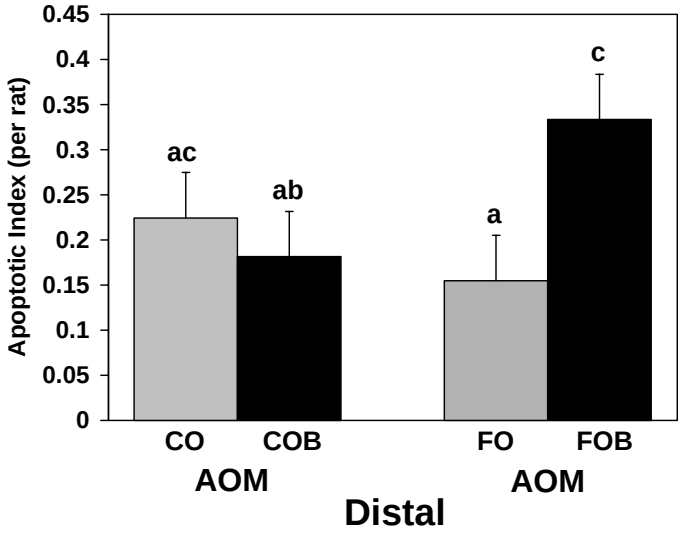

Figure 13 In rats injected with AOM, butyrate-supplemented diets enhanced apoptosis only in rats consuming fish oil. Apoptosis was measured in the distal colon only. Apoptosis is expressed as an apoptotic index (number of apoptotic cells / total number of cells per crypt column, x 100). Data are means ± SEM for 40 rats (n=10 rats per diet/treatment group). For each rat, 50 well-oriented crypt columns were assessed. Bars with different letters are significantly different (*P*<0.02). CO = corn oil; COB = corn oil+butyrate; FO = fish oil; FOB = fish oil+butyrate; AOM = azoxymethane.

Within fish oil+butyrate groups, there is an inverse relationship between the apoptotic index and the number of total ACF per rat ($P<0.05$; Figure 14). As the apoptotic index increases, the number of total ACF decreases; however this relationship is seen only in the fish oil+butyrate group, not in the corn oil+butyrate group (Figure 14).

p21$^{Waf1/Cip1}$ expression

Consistent with apoptosis, all assays investigating p21 expression were conducted in tissue from the distal colon. Within the crypt, regardless of diet or treatment, the intensity of p21 expression increases with each tertile (or, each one-third of the crypt), moving toward the lumenal surface. The lowest p21 expression is in the bottom tertile, with intermediate expression in the middle tertile, and the greatest expression in the top tertile ($P<0.0001$; Figure 15). In all non-butyrate supplemented rats, treatment with AOM decreased the expression of p21 compared to non-butyrate supplemented rats treated with saline ($P=0.0331$; Figure 16). However, when butyrate was provided, p21 levels did not decrease with AOM treatment ($P=0.0452$; Figure 16). Regardless of the lipid, butyrate supplementation maintained p21 expression levels similar to those of rats not exposed to carcinogen. Lipid had no effect on p21 expression.

Although rats consuming butyrate-supplemented diets experienced higher levels of both apoptosis and p21 expression individually, there was no direct relationship between p21 and apoptosis. However, in rats fed corn oil+butyrate, a positive

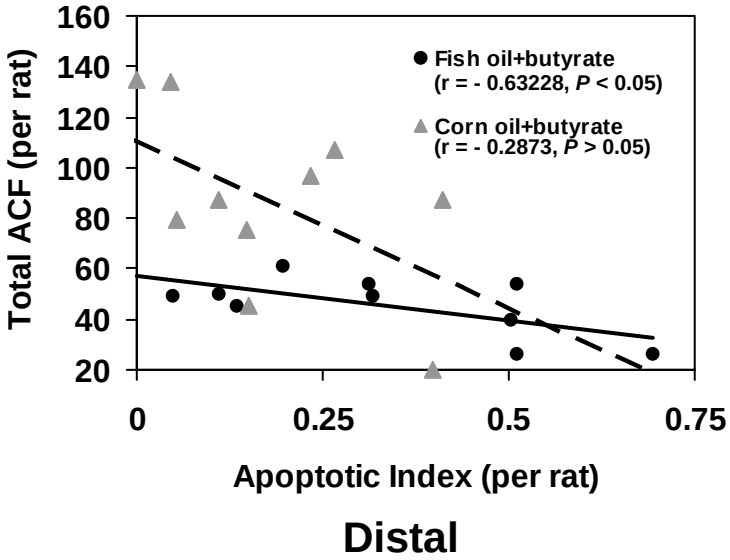

Figure 14 Among rats fed fish oil+butyrate diets (and injected with AOM), there is a negative relationship between the level of apoptosis and the number of total ACF. There is no relationship between rats fed corn oil+butyrate diets (and injected with AOM). Data are means ± SEM for 10 rats per group. ACF = aberrant crypt foci.

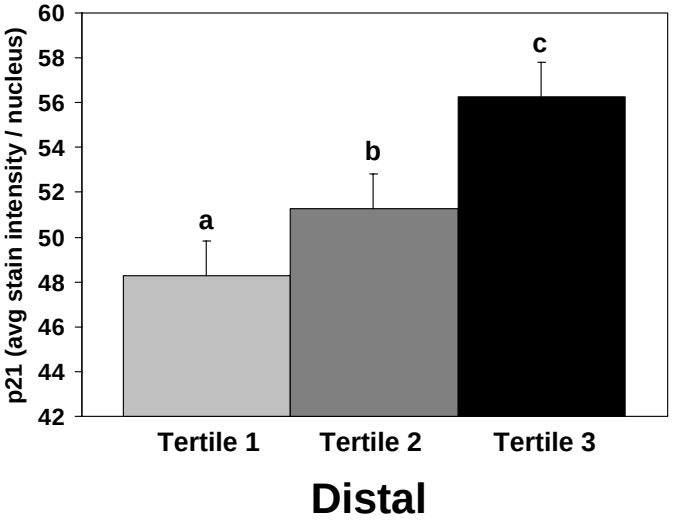

Figure 15 Regardless of diet or treatment, the expression of $p21^{Waf1/Cip1}$ (p21) increased as cells progressed up the crypt axis toward the lumen. p21 expression was lowest in the bottom tertile (1/3-crypt region), moderate in the middle tertile, and greatest in the top tertile. p21 was assessed in the distal colon only. Data are means ± SEM for 64 rats (n=64 rats per tertile). For each rat, 15 well-oriented crypt columns were selected and each nucleus circled to determine the mean p21 stain intensity per rat. Bars with different letters are significantly different (P<0.0001).

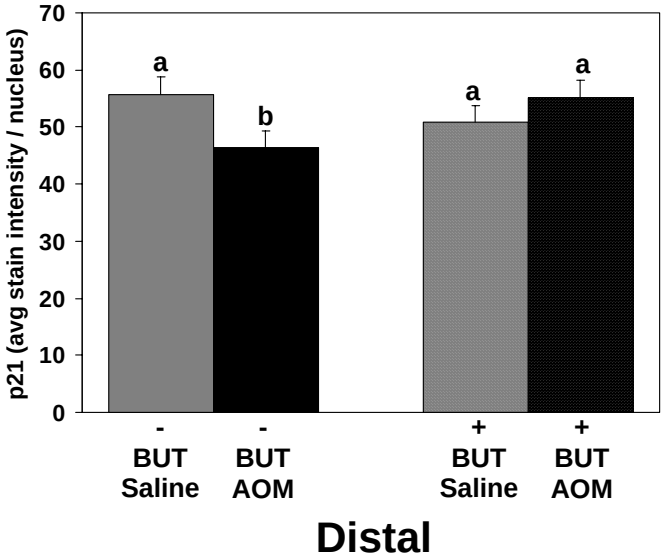

Figure 16 Among rats not consuming butyrate, AOM injection decreased p21 levels compared to saline injection, regardless of the lipid supplied. However, among rats injected with AOM, those consuming butyrate did not demonstrate the decreased levels of p21 seen in AOM injected rats not consuming butyrate. p21 was assessed in the distal colon only. Data are means ± SEM for 48 rats (n=16 rats per butyrate/treatment group). For each rat, 15 well-oriented crypt columns were selected and each nucleus circled to determine the mean p21 stain intensity per rat. Bars with different letters are significantly different (*P*<0.05). AOM = azoxymethane.

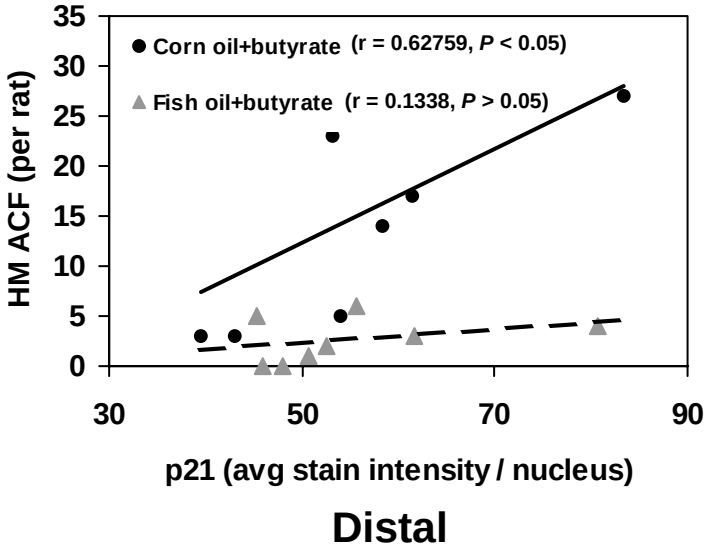

Figure 17 Among rats fed corn oil+butyrate diets (and injected with AOM), there is a positive relationship between the level of p21 expression and the number of HM ACF. There is no relationship among rats fed fish oil+butyrate diets. Data are means ± SEM for eight rats. HM ACF = high multiplicity aberrant crypt foci.

relationship was demonstrated between p21 expression and HM ACF in the distal colon ($P<0.05$; Figure 17). Rats consuming a corn oil+butyrate diet with enhanced p21 expression experienced greater numbers of HM ACF development; this relationship was not seen among rats fed fish oil+butyrate (Figure 17).

Histone H4 acetylation status

Consistent with both apoptosis and p21, all procedures investigating histone acetylation were conducted in distal colon tissue. Regardless of diet or treatment, the degree of histone H4 acetylation progressively decreases with each tertile moving toward the lumenal surface of the crypt. The greatest histone H4 acetylation occurs in the bottom tertile of the crypt, with less acetylation in the middle tertile, and the lowest degree of acetylation in the top tertile ($P<0.0001$; Figure 18).

Regardless of lipid, saline-injected rats consuming butyrate exhibited a 25% increase in histone H4 acetylation compared to saline-injected rats not consuming butyrate ($P=0.0104$; Figure 19). Alternately, with both AOM and butyrate treatments, dietary fish oil differentially modulates histone acetylation. In rats fed corn oil, there were no changes in histone acetylation status, regardless of AOM or butyrate treatment (Figure 20). However, in fish oil-fed rats injected with saline, butyrate supplementation enhanced histone acetylation, compared to that of non-butyrate fish-oil fed rats injected with saline ($P=0.0043$; Figure 21). In fish oil-fed rats consuming butyrate, those injected with AOM experienced decreased histone acetylation levels compared to those injected with saline ($P=0.0331$; Figure 21).

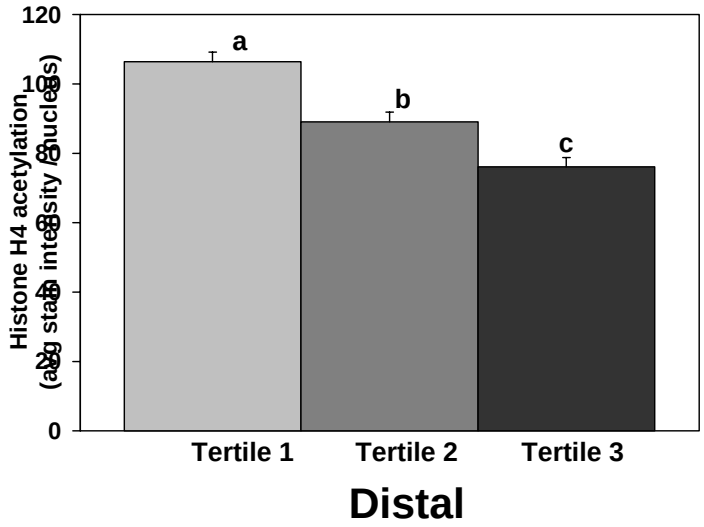

Figure 18 Regardless of diet or treatment, the expression of histone H4 acetylation decreased as cells progressed up the crypt axis toward the lumen. Histone H4 acetylation was greatest in the bottom tertile (1/3-crypt region), moderate in the middle tertile, and lowest in the top tertile. Histone H4 acetylation was assessed in the distal colon only. Data are means ± SEM for 64 rats (n=64 rats per tertile). For each rat, 15 well-oriented crypt columns were selected and each nucleus circled to determine the mean histone H4 acetylation stain intensity per rat. Bars with different letters are significantly different (*P*<0.0001).

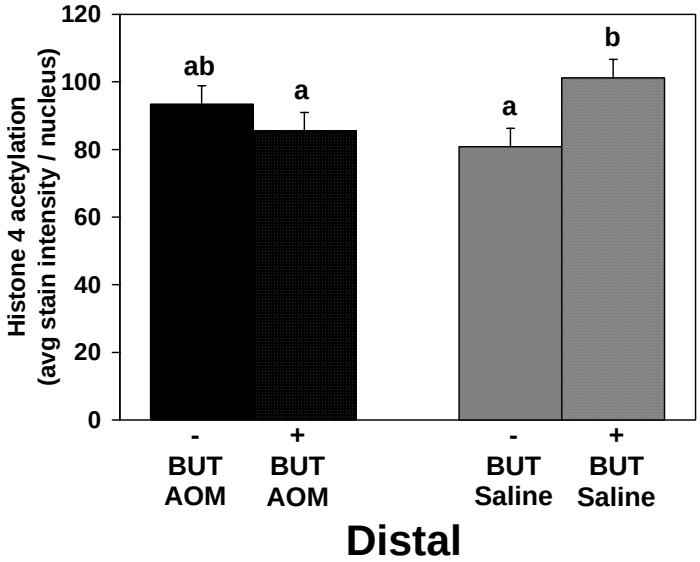

Figure 19 Regardless of lipid, dietary butyrate supplementation increased the level of histone H4 acetylation among saline-injected rats. There was no difference in acetylation status with or without butyrate supplementation among rats injected with AOM. Histone H4 acetylation was assessed in the distal colon only. Data are means ± SEM for 64 rats (n=8 rats per butyrate/treatment group). For each rat, 15 well-oriented crypt columns were selected and each nucleus circled to determine the mean histone H4 acetylation stain intensity per rat. Bars with different letters are significantly different (*P*<0.02). AOM = azoxymethane.

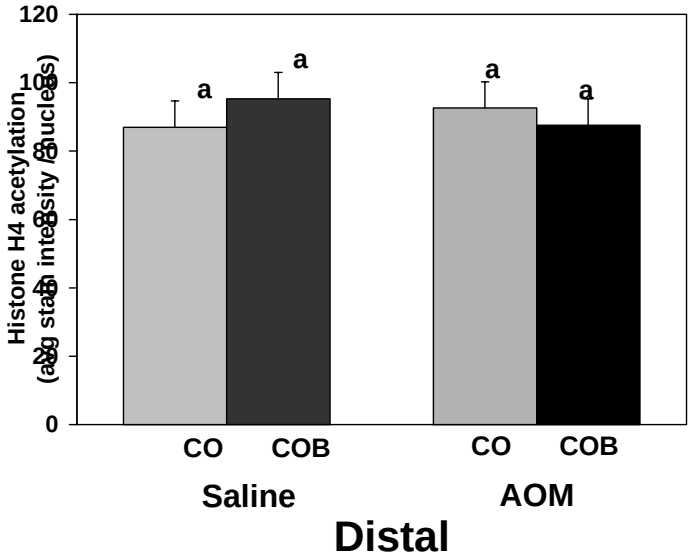

Figure 20 In the distal colon of rats fed corn oil, there were no changes in histone H4 acetylation, with either butyrate or injection treatments. Histone H4 acetylation was assessed in the distal colon only. Data are means ± SEM for 24 rats (n=eight rats per diet/treatment group; *P*>0.05). For each rat, 15 well-oriented crypt columns were selected and each nucleus circled to determine the mean histone H4 acetylation stain intensity per rat. CO = corn oil; COB = corn oil+butyrate; AOM = azoxymethane.

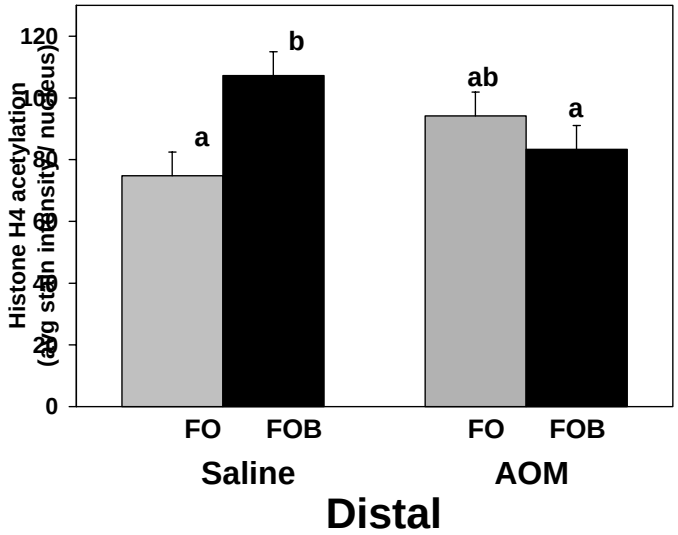

Figure 21 In the distal colon of rats fed fish oil and injected with saline, dietary butyrate increased the level of histone H4 acetylation. However, when rats were injected with AOM, fish oil+butyrate treatment decreased the level of histone acetylation as compared to fish oil+butyrate rats injected with saline. Data are means ± SEM for 24 rats (n=eight rats per diet/treatment group). For each rat, 15 well-oriented crypt columns were selected and each nucleus circled to determine the mean histone H4 acetylation stain intensity per rat. Bars with different letters are significantly different (*P*<0.04). FO = fish oil; FOB = fish oil+butyrate; AOM = azoxymethane.

CHAPTER IV

DISCUSSION AND CONCLUSION

Epidemiological context and previous findings support current hypotheses

The results from this study help to elucidate the mechanism for our general hypothesis that butyrate, a byproduct of pectin fermentation, protects against the promotion of colon carcinogenesis when supplied within a fish oil-rich lipid environment. Fish oil and fiber are independently hypothesized to be protective against colorectal cancer when consumed in the diet. For example, epidemiological studies documenting the incidence of colorectal cancer in Greenland Eskimos found that, compared to Western societies, this population develops significantly lower levels of the disease, which may be attributable to their consumption of native diets high in omega-3 PUFAs from fish (Byers, 1996). Similarly, native Japanese consuming traditional diets rich in fish have much lower rates of colon cancer than Japanese immigrants living in the United States who consume a traditional Western-style diet, which is low in omega-3 PUFAs (Bingham, 1998).

Dietary fiber was first linked to colon cancer prevention in the late 1960s when Dr. Dennis Burkitt published his landmark observations detailing the incidence of colorectal cancer among Aftrican tribespeople (Burkitt, 1969). He attributed their relative colorectal cancer-immunity to their native, high-fiber diet. Since that time, numerous reports have both challenged and supported his conclusions. For example, based on the Nurses Health Study of American women, Fuchs concluded that there is no protective effect of dietary fiber against colon cancer development (Fuchs *et al*, 1999).

Bingham, however, based on a cohort study of Europeans, observed that an increasing intake of fiber-rich foods is negatively correlated with colon cancer incidence (Bingham *et al*, 2003). Similar controversy currently surrounds the role of butyrate in colorectal cancer (Archer *et al*, 1998a; Caderni *et al*, 2001).

Individually, fish oil and fiber each appear to confer protection from colorectal cancer under certain conditions. However, we hypothesized that it may be an enhanced effect, observed when fish oil and a highly-fermentable fiber are consumed simultaneously, that affords the greatest protection. Previous studies in our lab have demonstrated that the dietary combination of fish oil and the highly-fermentable pectin fiber enhances their individual protective abilities. This combination results in an increase in apoptosis in a rodent model of carcinogen-induced colon cancer, when compared to the dietary combinations of fish oil and cellulose, a poorly-fermentable fiber, corn oil and cellulose, or corn oil and pectin (Chang *et al*, 1998). Although the specific mechanism(s) responsible for this interactive protective effect were not clear, we further hypothesized that it was butyrate, a byproduct of pectin's bacterial fermentation, within a fish oil-enriched lipid environment that mediates the protective effects seen with fish oil/pectin diets.

Therefore, in the present study, we tested the effects of feeding fish oil and butyrate, to begin detailing the mechanism(s) by which fish oil diets supplemented with pectin, rather than cellulose, enhance the protective effect of fish oil. By supplementing diets with butyrate, in the form of gastro-resistant sodium butyrate pellets targeted for degradation in the colon, we sought to produce a colonic lumenal environment similar to

that of a diet rich in a highly-fermentable fiber such as pectin. However, by supplying only the end-product of fiber fermentation, rather than the intact fiber itself, we have eliminated any other aspects of fiber that could potentially obscure our investigation of this unique metabolite's interaction with dietary lipid. Furthermore, by administering butyrate in a gastro-resistant, pelletized form, we minimized any confounding effects of butyrate manifesting within the GI tract prior to the colon.

While others have examined the effects of *in vivo* sodium butyrate supplementation, none have ever done so within the context of different specific lipid environments. Therefore, in this study, we demonstrate that butyrate's efficacy as an anti-cancer agent is dependent upon the broader lipid-context in which it is utilized. Employing a Sprague Dawley rat model, we examined the promotion stage of colon carcinogenesis within the context of four experimental diets: one corn oil-enriched and one fish oil-enriched diet, each supplemented with sodium butyrate pellets, and one corn oil-enriched and one fish oil-enriched diet, each without butyrate pellet supplementation. Within each diet group, 10 rats were injected with azoxymethane (AOM), a colon-specific carcinogen, and 10 rats were injected with saline, as a control.

Diet alters lumenal levels of total SCFA and butyrate

Although both are considered part of the large intestine, the proximal and distal colon regions respond differently to various treatments and stimuli, including diet. For example, we found that, of the total short chain fatty acids (SCFAs) that we measured (acetic, propionic, butyric, isobutyric, valeric, and isovaleric), proximal fecal material

contained a higher concentration of total SCFAs (µmoles/gwwf) than fecal material from the distal colon, similar to what we have previously shown (Zhang and Lupton, 1994). Each region also responds differently to dietary butyrate supplementation. Rats consuming butyrate had higher levels of total SCFAs (µmoles/gwwf) in the distal colon than their non-butyrate consuming counterparts, similar to the enhancement of total SCFAs that we have demonstrated with a highly-fermentable oat bran diet (Zoran *et al*, 1997b); however, when supplementing with butyrate, there was no difference between the concentration of total SCFAs produced with +butyrate or –butyrate diets within the proximal colon, unlike with oat bran supplementation. Nonetheless, these observations regarding the affect of diet on the concentration of total fecal SCFAs and fecal butyrate levels served to confirm that our model of dietary butyrate supplementation produced similar effects as a diet supplemented with intact fiber.

The manner in which diet modulates the concentration of fecal butyrate is particularly critical, as butyrate is important to many cellular activities. Therefore, the current investigation focuses on the concentration (mole %) of fecal butyrate, rather than any other single SCFA or total SCFAs, in the examination of how pectin confers its protective effects within a fish oil environment. More than any other SCFA, butyrate appears to possess unique properties within the cell, which have been demonstrated *in vitro* and *in vivo*. Butyrate treatment has reportedly induced cell cycle arrest (Archer *et al*, 1998a), cellular differentiation (Whitehead *et al*, 1986), histone hyperacetylation (Archer *et al*, 1998b), targeted gene transcription (Nakano *et al*, 1997), and apoptosis (Hague *et al*, 1993), in various cell lines and experimental models. Also, butyrate is the

preferred fuel source of the colonocyte (Roediger, 1980). Thus, it is clear that lumenal butyrate levels are vital to the cell.

Regardless of diet, the distal colon contained higher concentrations of fecal butyrate compared to the proximal colon. Although dietary butyrate supplementation alone did not appear to increase the concentration of fecal butyrate, we demonstrated that dietary butyrate supplied with fish oil, in fact, resulted in a significantly greater concentration of butyrate in the distal colon than does butyrate supplied with corn oil. Without butyrate supplementation, fish oil versus corn oil diets result in no differences in distal fecal butyrate concentrations. (We observed no effect of butyrate supplementation on fecal butyrate levels within the proximal colon.) Therefore, it appears that fish oil, perhaps by altering the bacterial flora within the colonic lumen, enhances the amount of butyrate to which the distal colonocytes are exposed; this idea is consistent with our previous findings, that both corn oil versus fish oil and cellulose versus pectin diets support different numbers of anaerobic fecal bacterial populations (Maciorowski *et al*, 1997). Whether or not the combined feeding of fish oil and pectin selects for a synergistically-protective microbial flora has not been evaluated, to date, although this could be another mechanism by which concomitant fish oil/pectin fiber consumption thwarts carcinogenesis.

Interestingly, the addition of a carcinogen significantly altered the level of fecal butyrate in the distal colon. Among saline-injected rats, those consuming fish oil+butyrate diets experienced greater fecal butyrate concentrations than those consuming corn oil+butyrate diets. However, among AOM-injected rats, there was no

difference in fecal butyrate levels between the fish oil+butyrate and corn oil+butyrate groups. AOM treatment appeared to blunt the increase of fecal butyrate seen in fish oil+butyrate rats. Unusual effects were also seen between carcinogen and butyrate treatments, regarding the distal fecal butyrate concentration in fish oil-consuming animals. AOM (versus saline) did not alter fecal butyrate levels in corn oil-fed rats, with or without butyrate. Likewise, AOM (versus saline) did not alter fecal butyrate levels in fish oil-fed rats that did not consume butyrate; however, in rats fed fish oil diets supplemented with butyrate, exposure to carcinogen significantly decreased fecal butyrate levels.

One possible explanation for this finding could be that the final step of AOM activation occurs in the colon (Fiala, 1977), and thus AOM metabolism here may either (1) significantly alter the bacterial flora cultivated by dietary fish oil and butyrate (Maciorowski *et al*, 1997), resulting in a decreased availability of butyrate to the colonocytes, compared with that which is available to saline injected rats; or AOM metabolism may (2) significantly alter (i.e., depress) the fish oil-enriched colonocyte's ability to utilize lumenal butyrate, compared to its saline-injected counterpart (Zhang *et al*, 1998). However, the mechanism(s) explaining why the bacterial flora and/or colonocytes of a rat consuming fish oil+butyrate would be more susceptible to AOM-treatment than any other diet is unknown.

The increase in fecal butyrate concentration leads to increased histone H4 acetylation

Butyrate has clearly been shown to act as an inhibitor of the histone deacetylase (HDAC) enzyme, leading to enhanced histone acetylation (Candido *et al*, 1978). Because histone hyperacetylation appears to be intimately related to enhanced gene expression, and because certain target genes (i.e., $p21^{Waf1/Cip1}$) could be effectors of butyrate, we measured the acetylation status of histone H4 as representative of butyrate's targeted histone acetylation and, potentially, subsequent gene expression (Nakano *et al*, 1997). Histone H4 is preferentially acetylated by histone acetyltransferases (Lahn *et al*, 2002), and it is strongly correlated with active gene transcription (Grunstein, 1997). Therefore, we hypothesized that by increasing the concentration of fecal butyrate in the lumen, butyrate supplementation would induce histone H4 hyperacetylation and that this may be one step through which pectin exerts its protective effects, in combination with fish oil. We also sought to determine if the degree of butyrate-induced histone H4 hyperacetylation varied within the crypt regions.

Here we show, in an unperturbed (non-carcinogen injected) state, that butyrate supplementation increased histone H4 acetylation by 25%; this is consistent with previous reports examining butyrate-induced histone acetylation in isolated cells via gel electrophoresis (Boffa *et al*, 1992). This technique, however, was unable to capitalize on the unique crypt architecture as a contextual tool for understanding potential differences in histone hyperacetylation within specific crypt regions. Therefore, we here demonstrate for the first time the pattern of histone H4 hyperacetylation within the

colonic crypt, as a function of dietary butyrate supplementation. The degree of histone acetylation is strongest in the bottom one-third (tertile) of the crypt, and decreases progressively with each tertile moving toward the lumen. This pattern mirrors the decreasing proliferation and increasing differentiation of the cells as they migrate up the crypt axis toward senescence at the lumen (Augenlicht *et al*, 1999b).

Histone acetylation resulting from *in vivo* butyrate supplementation can reflect multiple DNA-template activities within the cell, including replication as well as transcription (such as seen with the p21 protein) (Archer *et al*, 1998a; Kemp *et al*, 2005). The role of histone acetylation in replication may account for the increased histone acetylation that we observed in the lower, actively proliferating cells of the crypt, relative to those at the top of the crypt. Decreasing histone H4 acetylation patterns, such as we demonstrated in cells progressing upward through the crypt, could be reflective of an overall decrease in replication as cells move toward senescence. During replication, histone acetylation helps facilitate the ordered deposition of histone proteins onto newly synthesized DNA through a highly coordinated orchestration of protein complexes; HDACs remove these acetyl groups when the task of nucleosome building is complete (Akey and Luger, 2003). Further supporting the role of histone acetylation in replication, Kemp *et al* (2005) recently demonstrated that treatment of human cells with the HDAC inhibitor TSA results in hyperacetylation of histone H4 at multiple replication origins, enhancing the initiation of replication and the progression of cells through the S phase.

Interestingly, we found that carcinogen exposure decreased butyrate's ability to induce histone acetylation. In the presence of AOM, butyrate-induced histone acetylation was blunted, reduced to levels not significantly different from those of untreated rats (that is, saline-injected rats not consuming butyrate, with the lowest mean histone acetylation level). This may be reflective of the colonocyte's damage by AOM, rendering it unable to effectively utilize lumenal butyrate (Zhang *et al*, 1998).

Unexpectedly, dietary lipid appeared to modulate the manner in which AOM and butyrate affected the status of nuclear histone H4 acetylation. Histone acetylation levels of corn oil-fed rats appeared impervious to alteration, regardless of butyrate consumption or AOM injection; this is consistent with the concentration of fecal butyrate in corn oil fed rats, unaffected by butyrate or AOM. Unlike corn oil-fed rats, fish oil-fed rats experienced dramatic alterations in histone acetylation levels, due to both butyrate and AOM treatments. Butyrate treatment significantly enhanced histone acetylation in fish oil saline rats, compared to fish oil saline rats not consuming butyrate. This increase was not seen among corn oil-consuming rats. Likewise, in rats consuming butyrate, carcinogen treatment affected histone acetylation status as well. Fish oil+butyrate groups that were injected with AOM demonstrated a significant reduction in histone acetylation, compared to fish oil+butyrate groups injected with saline, a decrease not seen among corn oil-consuming rats. Again, this condition mirrors the suppressive effect of carcinogen on distal fecal butyrate levels in rats fed fish oil+butyrate.

An interesting trend was observed regarding changes in histone acetylation within corn oil and fish oil groups. Upon addition of butyrate to the corn oil-fed saline-

injected group, histone acetylation increased 9.7%, while adding butyrate to the corn oil-fed AOM-injected group decreased histone acetylation by 8.2%. However, upon addition of butyrate to the fish oil-fed saline-injected group, histone acetylation increased by a dramatic 30.3%, while adding butyrate to the fish oil-fed AOM-injected group decreased histone acetylation by 22.2%. While the increase and decrease in histone acetylation status is only significant in fish oil-consuming animals, this trend nonetheless demonstrates an interesting distinction between the manner in which these two lipid sources modulated the animal's response to butyrate and AOM.

Thus, the mechanism(s) controlling histone acetylation within corn oil-fed rats appeared strikingly insensitive to both butyrate and carcinogen treatments, while the mechanism(s) governing histone acetylation in fish oil-fed rats appeared highly sensitive to both butyrate and carcinogen treatments. Since this is the first investigation into dietary modulation of histone acetylation in the colon, very little preceding information is available to facilitate interpretation of these novel observations. From these data, it appears that dietary lipids could affect histone acetylation directly, via an unknown intracellular modality, or indirectly, by modulating the colonic lumenal environment and its response to butyrate and/or carcinogen. Therefore, the specific mechanism(s) behind such divergent responses warrant future investigation to elucidate the manner by which dietary lipids modulate nuclear histone acetylation, thereby influencing critical cellular processes.

The pattern of histone H4 acetylation is not reflective of, but complementary to, p21 expression

Butyrate has been clearly shown to induce expression of the cyclin-dependent kinase inhibitor p21, in various experimental models (Archer *et al*, 1998a; Litvak *et al*, 1998). From the current body of literature, it appears that butyrate induces p21 expression by acting as an HDAC inhibitor. Such HDAC inhibition enhances the acetylation of histones at the p21 gene promoter, making the region accessible to transcription factors and transcriptional machinery. Therefore, we expanded our hypothesis to include the idea that pectin's protective effects were mediated in part by p21, via butyrate's targeted histone hyperacetylation and consequential enhancement of $p21^{Waf1/Cip1}$ gene transcription. Thus, we expected the enhanced histone acetylation observed with butyrate treatment to result in a proportional increase of p21.

However, we found that the patterns of histone acetylation within the crypt were, in fact, opposite of those for p21 expression. As the level of histone acetylation progressively decreased moving upward through the tertiles, the expression of p21 progressively increased moving upward through the crypt. While at first glance these observations may seem incongruous, they are actually quite complementary. Just as the decreasing histone acetylation status is consistent with decreasing proliferation as cells migrate toward the lumen, the increasing expression of p21 corresponds with the documented progression of differentiation and apoptosis, as cells move upward along the crypt axis (Augenlicht *et al*, 1999b). p21 is well-established as an important mediator in butyrate-induced cell cycle arrest, purported to be a key step in the cell's transition from

active proliferation to differentiation (Archer *et al*, 1998a). Furthermore, the cell's evolution from an actively proliferating state to a quiescent, differentiated state is typically required for apoptosis, which is an expected event as cells reach the upper crypt region (Augenlicht *et al*, 1999b). Thus, the two distinctive patterns appear to reflect different, but corresponding, cellular activities within the crypt.

Such opposing patterns seem to denounce the possibility that the observed increase in p21 expression is attributable to a proportional increase in histone acetylation. However, it is nonetheless conceivable that butyrate-enhanced p21 expression does, in fact, depend upon targeted histone hyperacetylation (via butyrate's HDAC-inhibition), but that this relatively subtle enhancement of histone H4 acetylation at the p21 locus is not detectible at the genomic level. Because our current methodology identified all acetylated histone H4 proteins within the nucleus, rather than only those isolated to the p21 promoter region, the status of histone H4 acetylation that we detected could reflect many activities within the cell, beyond that of *p21* gene expression. While this global histone H4 acetylation may be valuable in assessing where the cell is in its life cycle, it may overshadow the more subtle changes in acetylation that might be seen at specific gene loci. Thus, by our current methods, it cannot be demonstrated conclusively whether or not butyrate increased p21 expression by enhancement of histone acetylation. Further efforts to isolate the p21 promoter region and measure its individual status of histone acetylation in response to various diet treatments are warranted.

Butyrate treatment enhances both p21 expression and apoptosis

Although the specific functions of p21 *in vivo* are controversial, we hypothesized that increased expression of p21, within a fish oil-rich environment, would increase the cell's apoptotic response to carcinogen-induced mutation at the promotion stage, thus protecting the colon against neoplasia. Although we cannot say at this time whether butyrate induced p21 by the manner we proposed, it is nevertheless clear that dietary butyrate enhanced the expression of p21 during a carcinogen insult, and that this enhancement was not dependent upon the lipid source.

While some studies have labeled p21 as anti-apoptotic (Gorospe *et al*, 1997; Wang and Walsh, 1996), other evidence suggests that p21 induces cell cycle arrest and apoptosis, and that both are potentially protective against colon cancer. Archer *et al* (1998a) found that p21 is necessary for cell cycle arrest in response to butyrate treatment, as well as for the cell's transition toward differentiation. Yang *et al* (2001) demonstrated that the loss of p21 results in decreased apoptosis and increased proliferation in a murine colon cancer model; likewise, Duttaroy *et al* (1997) found that apoptosis is dependent upon functional p21, as transfection of mouse 3T3 fibroblasts with antisense p21 prevented apoptosis.

Here, we demonstrated, in the absence of DNA damage (i.e., no AOM) and thus no need for cell cycle arrest or apoptosis, that butyrate supplementation did not increase p21 expression. Upon carcinogen (AOM) administration, however, intrinsic p21 levels were severely diminished—that is, the non-butyrate treated, AOM-injected rats had lower p21 levels than the non-butyrate treated rats injected with saline. When butyrate

was supplemented in the diet, rats injected with AOM had higher levels of p21 than non-butyrate supplemented rats injected with AOM. Butyrate treatment was able to offset the effects of AOM, maintaining p21 expression at a level not statistically different from that of untreated animals not exposed to carcinogen.

While we did not demonstrate a direct relationship between p21 protein expression and apoptosis, we did find that apoptosis was enhanced with butyrate treatment, and that this enhancement occurred only when butyrate was supplied in combination with fish oil. Although fish oil induced apoptosis independent of butyrate treatment, the combination of dietary fish oil+butyrate increased apoptosis more than dietary fish oil alone. In fact, the fish oil+butyrate diet resulted in more apoptosis than the corn oil+butyrate diet, in both AOM and saline treated rats. This is consistent with our previous findings, that fish oil+pectin diets result in the highest apoptotic index, while corn+pectin diets result in the lowest (Chang *et al*, 1997).

Since we did not find a direct correlation between p21 and apoptosis, it is possible that butyrate's enhancement of the apoptotic cascade within the colon may occur through a mechanism(s) that does not depend directly upon p21 as a definitive mediator. In fact, the role of p21 in facilitating apoptosis may simply be preliminary: when upregulated by butyrate, p21 may function as the inducer of cell cycle arrest, thus poising the cell for a butyrate-mediated apoptotic response to carcinogen.

However, while p21 may act as an early preparatory agent in butyrate's induction of apoptosis, it may actually serve dual functions. While further examination is needed to ascertain the exact role of p21 in the butyrate-mediated apoptotic cascade, it is clear

that butyrate treatment does increase p21 and caspase-3 expression (Archer *et al*, 1998a; Newton *et al*, 2004), both of which have been linked to apoptosis. As previously mentioned, Archer *et al* (1998a) found that p21 was required for butyrate-mediated growth arrest. Zhang *et al* (1999) found that caspase-3 mediated cleavage of p21 was necessary to convert human lung carcinoma cells from growth arrest to active apoptosis. Furthermore, during apoptosis in LIM 1215 colorectal cancer cells, Chai *et al* (2000a) reported that caspase-3 cleavage of p21 produced a unique 15 kD protein fragment. It is unknown at this time whether p21 cleavage inactivates a potentially anti-apoptotic p21 protein and thereby allows the apoptotic cascade to progress, or if p21 serves as the substrate for an important pro-apoptotic protein fragment. In either case, it may be that p21 enhancement by butyrate is necessary to induce cell cycle arrest in response to carcinogen treatment, and that subsequent to cell cycle arrest the butyrate-enhanced caspases are responsible for cleaving p21, thus committing the cell to apoptosis. In retrospect, it may be that a p21-protein cleavage product is positively correlated with apoptosis in fish oil+butyrate fed animals, rather than the whole p21 protein itself; this is another mechanism that warrants future investigation to further elucidate the apoptotic pathway in response to diet.

Butyrate-mediated apoptosis leads to decreased aberrant crypt formation

We hypothesized that both total ACF and high multiplicity ACF (HM ACF) would be lower in rats consuming fish oil+butyrate diets as a result of butyrate's enhancement of apoptosis. Consistent with this hypothesis, we found that the fish oil+butyrate diet

resulted in the fewest HM ACF compared to all other diets, and the fewest total ACF compared to the corn oil+butyrate diet. We also demonstrated for the first time that the corn oil+butyrate diet generated the highest numbers of both total and HM ACF. These findings parallel the divergent apoptotic responses seen between the two diets, with fish oil+butyrate resulting in the most apoptosis and corn oil+butyrate resulting in the least apoptosis of all four diet groups. Moreover, only in rats fed a fish oil+butyrate diet was the level of total ACF and apoptosis inversely related; among these rats, as the apoptotic index increased the number of total ACF decreased. This relationship was not seen in rats fed a corn oil+butyrate diet. It thus appears that one mechanism by which a fish oil+butyrate diet protects against the development of aberrant crypts is via an increase in apoptosis. The high levels of HM ACF seen with a corn oil+butyrate diet implicate it as potentially promotive of colon tumor formation. Therefore, our results with corn oil+butyrate diets are consistent with that found by Caderni, who reported that dietary butyrate supplementation did not protect against ACF development, within their high fat corn oil diet model (Caderni *et al*, 2001).

It may be that that butyrate-enhanced p21 and caspase-3 are two key regulators of the fish oil+butyrate-mediated apoptotic pathway, and that this occurs in a cooperative fashion; however, greater examination is needed to understand the specific mechanism(s) of this relationship. This hypothesis could help explain why increased p21 expression in response to AOM and butyrate treatment was positively correlated with HM ACF in corn oil-fed rats, but not in fish oil-fed rats. We have previously shown that caspase-3 activity is enhanced with butyrate treatment, but *only* in rats

consuming fish oil (Newton *et al*, 2004). Butyrate treatment increased caspase-3 activity 17.4% in fish oil-fed rats (P=0.04), while butyrate treatment had no effect on caspase-3 in corn oil-fed rats (Newton *et al*, 2004). This corroborates the hypothesis that butyrate-enhanced p21 expression is important to apoptosis, and is thus protective against colon carcinogenesis, but only within a fish oil-enriched environment where butyrate treatment simultaneously increases caspase activity. An increase in p21 expression with butyrate treatment may be deleterious to the cell if there is no concomitant increase in caspase-3 to cleave p21, such as in the case of corn-oil fed rats. This may explain why rats fed corn oil+butyrate and injected with AOM experienced enhanced HM ACF development, and why this dietary combination alone demonstrated a positive correlation between p21 and HM ACF. These data suggest that corn oil+butyrate and/or corn oil+pectin diets are actually more promotive of carcinogenesis than a high-fat corn oil diet alone, without fiber or butyrate supplementation.

Conclusion

We found that a diet rich in fish oil and supplemented with butyrate was the most effective diet treatment in the prevention of both increased numbers and multiplicity of carcinogen-induced precancerous colon lesions. Using total ACF and HM ACF as end phenotypes, we demonstrated that dietary butyrate plays a critical role in colon carcinogenesis—but that this role is modulated by the specific dietary lipid source.

Consistent with our original hypothesis, we found that it was the specific lipid provided in the diet that was responsible for the degree of protection against

carcinogenesis. Contrary to what one might expect, the decrease in p21 with AOM-injection and without butyrate supplementation did not place the +AOM, –butyrate rats at the highest risk for cancer. Instead, AOM-injected rats fed corn oil and fish oil diets with no butyrate supplementation did not differ in apoptosis (P=0.3302; data not shown), total ACF, or HM ACF, placing them at intermediate risk of colon carcinogenesis compared to the corn oil+butyrate and fish oil+butyrate diet groups.

Despite an increase in p21, AOM-treated, butyrate-supplemented rats demonstrated the greatest disparity in colon cancer protection, which was contingent upon the lipid group provided. Interestingly, the increase in p21 observed in corn oil+butyrate rats injected with AOM did not result in a corresponding increase in apoptosis; rather, an attenuation of apoptosis resulted. Moreover, the elevated p21 levels within the corn oil+butyrate group were directly related to pre-cancerous lesion formation. Through an unknown mechanism, elevated p21 levels within a corn oil-enriched environment appear to be a risk factor for HM ACF formation. Furthermore, it appears that the protective effect of the fish oil+butyrate diet is due to the unique properties of fish oil, providing an environment in which butyrate's enhancement of histone acetylation and p21 expression are pro-apoptotic. As a result of enhanced apoptosis, there is a decrease in pre-neoplastic lesion formation in the colon when fish oil and butyrate are concomitantly consumed.

The present work, therefore, helps advance the field of colon cancer prevention by: (1) helping to clarify the apparent contradictions seen between various studies that support and deny the protective role of butyrate (or fiber) in colon cancer; (2) providing

evidence to help resolve the controversy surrounding the function of $p21^{Waf1/Cip1}$ *in vivo*, demonstrating for the first time that the pro- or anti-apoptotic properties of p21 may depend upon the specific diet consumed; (3) documenting portions of the butyrate-induced apoptotic pathway and providing preliminary data for future investigations; and by (4) demonstrating for the first time the expression of acetylated histone H4 protein and the $p21^{Waf1/Cip1}$ protein *in situ* within the colonic crypt, as a function of dietary lipid and butyrate consumption, and how their expression relates to apoptosis. Furthermore, this work supports the concept that rather than supplementing the diet with one single "protective" compound, the whole diet must be taken into consideration for optimal health and disease prevention.

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APPENDIX A

EXPERIMENTAL DIET MIXING PROTOCOL

(Anne Newton, 2/2002)

For mixing 200 kg (8 batches) of dry Teklab basal mix—85% basal mix, 15% fat:

1. **Take oil (fish) out of freezer 24 hours prior to mixing and allow it to thaw.**
2. **Gather supplies for mixing diets and take to basement:**

   (2) Large mixing bowls for Hobart mixer (found in Lupton lab)

   (2) Beaters for Hobart mixer (found in Lupton lab)

   Pre-labeled 1-gallon Ziploc bags (approx. 1 kg of diet per bag)

   Small Rubbermaid weighing bins

   (3) Scoops

   70% EtOH in spray bottle for clean-up

   Box of gloves, Paper towels, Scissors, Colored labeling tape, Sharpies, dust masks

   "Lid" to mixing bowl (the trash can lid with the hole in it)
3. **Gather supplies for mixing oils and load onto large cart for transport to Chapkin lab:**

   (10) 5000 ml plastic beakers

   Pkg 25 ml sterile pipettes

   Pkg 10 ml sterile pipettes

   (2) Automatic pipettes

Absorbant diapers

Paper towels

4 large plastic bins full of ice

To mix the oils (Corn first):

(Corn oil, Tenox and MTS-70 are stored in 4°C refrigerator in Dr. Chapkin's lab)

a. Use the top-loading scale in Dr. Chapkin's lab

b. Bubble the scale

c. Place an empty 5000 ml beaker on the scale and tare

d. Label the beaker (corn or fish oil)

e. Add the appropriate weight of corn oil to the beaker (Use the 2 extra beakers as "overflow" beakers to remove any excess oils during the measuring process)

f. Repeat steps c-e 3 times for a total of 4 beakers of corn oil

g. Next, weigh out the fish oil in the same manner as the corn oil. Then add the appropriate amount of corn oil to the fish oil. Repeat for a total of 4 beakers of fish oil.

h. To each beaker of oil, add the appropriate weights of Tenox 20A and MTS-70 using sterile pipettes After each addition, stir the oil with a sterile pipette for one minute or until well mixed

i. Cover the beakers with foil and pack in ice until use

j. Flush original stock containers of oil with nitrogen and return them to refrigerator or freezer

4. Measuring the dry mix:

a. To the basement, bring the 8 beakers of ice-packed oil, all above listed supplies, and the top-loading scale from Dr. Chapkin's lab.

b. Make sure both doors to the mixing room are shut!

c. Re-bubble the scale

d. Make sure the bowls and mixing beakers are adequately labeled with colored tape and Sharpies to avoid confusion!

e. Place one Rubbermaid bin on the scale and tare

f. Begin to scoop the dry premix from the barrel into the bin, trying to stir up as little dust as possible (gloved hands are best throughout the mixing process)

g. Weigh out 5000 g and pour into the large mixing bowl labeled for corn oil diet. Repeat for a total of 25000 g (the barrel should be all but empty)

5. Mixing the diets:

a. Attach the bowl of dry mix

b. Attach the beater labeled for corn oil diets to the Hobart mixer and raise the bowl (use crank)

c. Thoroughly stir one of the beakers of corn oil and pour about ½ of the oil into the bowl of dry mix.

d. Cover the bowl with the "lid" and mix on setting 1 for two minutes (until the oil has blended in)

e. Add the remaining oil and continue to mix on setting 1 until blended (about 5 minutes)

f. Stop the mixer and lower the bowl. Thoroughly scrap the beater using gloved hands. Raise the bowl

g. Mix on setting 2 for 10 minutes

During the mixing period, begin measuring the dry mix as directed above and pour into the bowl labeled for fish oil diet. When the corn oil diet is finished mixing, remove the bowl and scrape the beater. Attach the fish oil beater and bowl and mix as directed above. Bag the mixed corn oil diet, wipe out the bowl and begin the measuring process once again.

6. To bag the diets:

a. Fill the pre-labeled Ziploc bags about 2/3 full using the scoop labeled for that particular diet

b. Push the air from the bags and seal, and then place the sealed bag into a freezer bag. Remove the air and seal. Pack into boxes or barrels. Store in -20°C freezer

7. Clean up:

a. Wash the bowls, beaters, scoops, bins, beakers, etc in the large sink and place on the straining rack to dry.

b. Sweep and mop the floor and wipe down all surfaces with 70% EtOH and a paper towel

c. When the bowls and beaters are dry, wipe down with 70% EtOH

d. Return all equipment

APPENDIX B

48-HOUR FOOD INTAKE PROTOCOL

(Stella Taddeo, 7/2002)

DAY 1

1. Record rat number on form.
2. Remove old food cup and food.
3. Put Xerox paper under rat cage.
4. Weigh and record weight of empty food cup.
5. Add approximately 35 g designated experimental diet to cup.
6. Record total weight of cup and food.
7. Put the cup in the rat cage.

DAY 2

1. Start with the first rat from day one and proceed in the same order.
2. Tare the balance.
3. Remove the food cup from the cage and put the cup on the balance.
4. Carefully remove the paper from under the cage.
5. Pick off any fecal pellets and discard (wear gloves).
6. Dump the spilled food from the paper into the cup.
7. Record the weight of the "leftover cup &food + spill" on the form under "DAY 2".
8. Tare the balance.
9. Put a clean cup on the balance.

10. Record the weight of the clean cup under DAY 2, new cup wt.

11. Add approximately 35 g designated experimental diet to the cup.

12. Record the total weight of the cup + the food.

13. Put the cup in the cage

14. Weigh the rat and record his weight.

15. Return the rat to his cage.

DAY 3

1. Start with the first rat from day one and proceed in the same order.
2. Tare the balance.
3. Remove the food cup from the cage and put the cup on the balance.
4. Carefully remove the paper from under the cage.
5. Pick off any fecal pellets and discard (wear gloves).
6. Dump the spilled food from the paper into the cup.
7. Record the weight of the "leftover cup &food + spill" on the form under "DAY 3."
8. Weigh the rat and record his weight.
9. Return the rat to his cage.

APPENDIX C

ABERRANT CRYPT FOCI ENUMERATION PROTOCOL

(Cindy Warren, 2001. Modifications by Kristy Covert, 8/2002)

Supplies:

Methelyne blue (recipe follows) in clear specimen cup

PBS in clear specimen cup

Rubber gloves

Long stem cotton swabs

Labeled tissue samples in clear specimen cup

Clear grid (with ½ cm squares)

ACF score sheets

Zip disk

Paper towels

Clip board

Procedure:

1. Place all supplies in small cooler with waffle lab bench paper in the bottom.
2. In the microscope room, turn on the four switches to prepare to use the microscope (computer, scope, TV screen, grey box).
3. Stain the tissue by dipping in the methylene blue for 12-15 seconds. If too dark dip it back into the ethanol.
4. Place on plastic grid taped onto microscope stage, beneath lens, 10X

magnification. Take care not to touch the lens.

5. Note rat ID number, length of colon, date, etc. on the score sheet.

6. Starting on the distal end (this end doesn't have tissue architecture with ridges). Go slowly through each box, examining the tissue.

7. Note any ACF, both position and multiplicity.

   Normal crypts are oval or round and a lighter blue stain.

   ACF are darker and have swirl-like appearance.

   Many of the ACF have increased size, thicker epithelial lining, and darker staining luminal openings.

   Compare the ACF to the surrounding tissue.

   Hyperproliferative crypts are not as distorted as the ACF.

   ACF have a larger white, distorted zone in the luminal opening.

   Peyer's patches are lymphatic tissue; look like big cloudy spots

8. Make sure that tissue does not dry out—using cotton swap, moisten tissue periodically with PBS. It should remain shiny.

9. **This procedure is totally subjective.** Determine individual standards for scoring ACF and remain consistent with each tissue.

APPENDIX D

SHORT CHAIN FATTY ACID GAS CHROMATOGRAPHY ANALYSIS PROTOCOL

(Kristy L. Covert, 12/2002)

Part I—Preparation of Sample

Preparing Instruments:

1. Lay out bench papers (one per sample) and foil squares (one per sample); have paper towels and kim wipes nearby.
2. Pour two large buckets of liquid nitrogen; place lids on buckets.
3. Gather two ceramic mortar/pestle sets, several small metal spatulas, and tongs; place into liquid N (they need to be very cold).
4. Place empty labeled 1.8 ml cryotubes in liquid N.
5. Remove fecal samples from freezer—place in liquid N.

Grinding Sample—the following should all be done quickly, taking care to keep the samples and instruments very cold:

6. Wearing gloves, remove first fecal sample from liquid N; open tube and dump contents onto foil square.
7. Fold foil around sample (into a "packet"), and submerge in liquid N for several seconds.
8. Remove packet and pound several times with hammer.
9. With tongs, remove mortar from liquid N; leave ¼ full of liquid N.

10. Carefully open packet and pour fecal sample into mortar (take care to not lose any sample!).
11. With tongs, remove pestle from liquid N; wrap paper towel around pestle and grind sample until liquid N evaporates and/or sample is completely powdery. (You may add a ziplock bag "skirt" around pestle and covering mortar to prevent sample flying into air during grinding.)
12. If necessary, add some more liquid N and continue grinding.
13. While waiting for remaining liquid N to evaporate, wipe off pestle with paper towel and put back into liquid N bucket.

Transferring powdered sample:

14. Remove labeled cryotube; remove lid and place tube into rack (submerged in liquid N).
15. With cold spatula, carefully scoop powdered sample into cryotube, dipping spatula into liquid N frequently (to prevent sample from sticking to spatula).
16. When sample completely transferred to tube, place lid on tube and return to liquid N bucket.
17. Wipe off mortar and spatulas; return to liquid N bucket.
18. Carefully fold bench paper and discard; clean up work area to prepare for next sample.

Part II—Preparation of Sample, Day Before GC Analysis

1. Label 1.8 ml eppitubes with sample identification number

2. Get bucket of ice (to set tubes down in)
3. Fill red liquid N canister, blue liquid N canister, and a rectangular tub with liquid N; take to balance
4. Put 10 ml glass beaker onto balance
5. Chill one eppitube in liquid N; place in beaker on balance and TARE
6. Chill metal spatula (I like the one with the red handle best); transfer frozen powdered fecal material from sample tube to eppitube
7. Record weight, to the thousandth place (ideal weight: 0.35 – 0.37 grams of fecal material)
8. Set eppitube in ice; if weight was 0.2 g or above, add 1 ml of 0.511 mM 2-ethyl butyric acid standard; if weight was below 0.2 g, add only 0.5 ml of 2-ethyl butyric acid standard
9. Let eppitube sit in ice, with cap open, during the preparation of the next sample (this allows time for any gas to escape)
10. Add standard to the second tube, and let it sit in ice; close the previous tube and vortex approximately 15 seconds (mix thoroughly)
11. Place closed eppitube down into ice
12. Repeat with subsequent samples
13. When finished, vortex all samples again, and place in 4º refrigerator overnight

Part III—Preparation of Sample, Day Of GC Analysis

1. Start the fast cooling of microcentrifuge

2. Remove sample (in 1.8 mleppitube) from refrigerator
3. Vortex eppitube for approximately 10 seconds
4. Put eppitube in rotating shaker; shake for 20 minutes
5. Remove eppitubes; vortex for approximately 5 seconds
6. Place eppitubes in microcentrifuge with hinges at top, and balance with balance tubes; spin for 20 minutes at 15,000 RCF at 4º
7. During the spin, get ice in small bucket
8. Remove eppitube from microcentrifuge GENTLY, so as to not upset the supernatant; place tubes in ice
9. With a 200 µl pipette, draw out the supernatant and put into 2 ml glass GC tubes (with green cap)
10. Place GC tubes with supernatant in ice
11. Immediately prior to GC analysis, remove 200 µl supernatant and place in 2 ml glass GC tube (with red cap)
12. Add 200 µl of 0.830 mM heptanoic acid
13. Add 20 µl of 10% ortho-phosphoric acid
14. Therefore, what actually goes onto the GC is: fecal sample supernatant, 0.256 mM 2-ethylbutyric acid, 0.415 mM heptanoic acid, and ortho-phosphoric acid

APPENDIX E

APOPTOSIS ASSAY PROTOCOL

(From Intergen. Modifications by Wen-Chi Chang and April Carney, 4/28/99.

Modifications by Kristy Covert 7/2003)

1. Prep:
   - Use thick slide holder (it works better)
   - Prep humid chambers; put one in warmer, leave one at RT
   - Rinse bleached items
   - Mark positive control slide with red "X" (last section will be negative control)
   - Measure 200 ml PBS (for Prot. K) in boat; put in 37º C oven
2. Deparaffinize in Xylene
   - 5 min.
   - 5 min.
   - 5 min.

     Jiggle slides around, to make sure they don't stick to each other
3. Rehydrate
   - 100% EtOH—5 min.
   - 100% EtOH—5 min.
   - 95% EtOH—3 min.
   - 95% EtOH—3 min.
   - 70% EtOH—3 min.
   - PBS—5 min.

4. During above wash, take 110 µl aliquot of Proteinase K
   - Quickly return Prot. K to freezer—it MUST stay cold!
   - Let aliquot warm to RT
5. Circle tissues w/ Pap pen—do quickly, so tissues don't dry out!
6. Take DAB out of freezer, put in drawer to thaw
7. Pre-treat Tissue w/ Proteinase K
   - 200 ml warmed PBS + 100 µl Prot. K (10 µg/ml PBS); stir
   - Incubate 6 min. @ RT
8. Wash in dH2O
   - dH2O—2 min.
   - dH2O—2 min.
9. Thaw DNase Buffer, Equilibration Buffer, & Reaction Buffer @ RT
10. Quench Endogenous Peroxidase
    - 198 ml methanol + 2 ml 30% H2O2; stir
    - 30 minutes @ RT
11. Wash in dH2O
    - dH2O—5 min.
    - dH2O—5 min.
12. During above washes, take aliquot of Equilibration Buffer and warm to RT

    (65 µl X # of sections)
13. During above washes,
    - 120 µl sterile water + 15 µl DNase buffer

- Vortex, put on ice; return DNase buffer to freezer
- Get DNase from freezer; put in ice

14. POSITIVE CONTROL (Put all other slides in PBS)
    - Immediately before applying, add 15 µl Dnase 1 to the sterile H2O/Dnase buffer
    - Incubate 5 min. @ RT in pre-warmed humid chamber

      (After incubation, separately rinse positive control with PBS)
15. Wash in PBS
    - 5 min.
16. Gently tap off PBS and carefully blot around sections.
17. Apply room-temp. EQUILIBRATION BUFFER to ALL sections
    - Incubate in open air 15 sec to 1 hr. @ RT
18. Dilute TDT with Reaction Buffer
    - 1 part TDT + 8 parts Reaction Buffer (a 1:8 ratio)

      (65 µl X # of sections)
    - Vortex, put on ice; put TDT back in freezer
    - Immediately apply diluted TDT to all sections, except for negative control section (last section on pos. control slide gets Reaction Buffer ONLY)
    - Incubate in pre-warmed humid chamber 1 hr. @ RT
19. During above incubation, pour working strength Stop/Wash Buffer into Coplin Jar; allow to come to RT
20. Put slides in Stop/Wash Buffer (Put slides back-to-back—2 per slot)
    - Agitate 15 sec.

- Incubate 10 min. @ RT

21. During above incubation, aliquot Anti-Digoxigenin Peroxidase
    - 65 µl X # of sections
    - Allow to come to RT
22. Wash in PBS
    - 1 min.
    - 1 min.
    - 1 min.
23. Apply Anti-Digoxigenin Peroxidase to ALL sections
    - Incubate 30 min. in humid chamber @ RT
    - Each slide should receive EXACTLY 30 minutes of incubation
24. Wash in PBS
    - 2 min.
    - 2 min.
    - 2 min.
    - 2 min.
25. During above rinses, prepare 0.05% DAB solution (0.5 mg/ml)
    - 198 ml PBS + 2 ml stock DAB in beaker
    - Filter through Whatman #1 paper
    - IMMEDIATELY before staining, add 100 µl 30% H2O2!!
    - Stain for 20 sec.
26. Wash in dH2O

- 1 min.
- 1 min.
- 1 min.
- 5 min.

27. Counterstain with 0.05% Methyl Green
    - Dip quickly in Methyl Green
28. Immediately rinse in dH2O
    - Dip quickly
    - Dip quickly
    - Dip quickly
    - Dip 10 times, then let set for 30 sec.
    - 1 min.
    - 1 min.
29. Dehydrate
    - 70% EtOH—1min.
    - 95% EtOH—1 min.
    - 100% EtOH—1 min.
    - Xylene—2 min. (dip 10 times each)
    - Xylene—2 min. (dip 10 times each)
    - Xylene—2 min. (dip 10 times each)
30. Mount with Permount and Coverslip.

Reagent	Company	Catalog #
Apotag Kit:*	Intergen	S7101
Proteinase K	Ambion	2546
DNase 1	Ambion	2222
PBS	Life Technologies	21600-069
DAB	Sigma	D5637
Permount	Fisher	SP15-100

*As of 2/19/02 the kit parts are good for 3yrs WITHOUT thawing. The Equilibration buffer is good for 4 years without thawing.

APPENDIX F

P21 $^{Waf1/Cip1}$ DETECTION ASSAY PROTOCOL

(Kristy L. Covert, 2/2003)

For use with 4 micron sections of 4% PFA-fixed, paraffin-embedded rat colon tissue

1. Deparaffinize slides in xylene
   - ❑ **5 min.**
   - ❑ **5 min.**
   - ❑ **5 min.**
2. Rehydrate in graded alcohols
   - ❑ 100%, **2 min.**
   - ❑ 100%, **2 min.**
   - ❑ 95%, **2 min.**
   - ❑ 95%, **2 min.**
   - ❑ 70%, **2 min.**
3. Wash in PBS, **5 min.**
4. Quench endogenous peroxidase

   250 ml glass boat, add **<u>180 ml methanol + 20 ml 30% H2O2</u>**, for 200 ml total dilute solution—add H2O2 immediately before using

   - ❑ 3% H2O2, **30 min.** @ RT
5. Rinse in PBS
   - ❑ **5 min.**

<u>Run Info</u>

Date: _____

Start Time: _____
Time Into 4º: _____
Time Out of 4º: _____
Finish Time: _____

Slide #1: _____
Slide #2: _____
Slide #3: _____
Slide #4: _____
Slide #5: _____
Slide #6: _____
Slide #7: _____
Slide #8: _____

- **5 min.**

6. During above second rinse, warm diluted Retrievit antigen retrieval solution
    - **3 min. on HIGH (or until it comes to rolling boil)**
7. Antigen Retrieval
    - Place 3 slides (per plastic Coplin jar) in warm Retrievit solution (add a blank slide, if necessary)
    - Include one extra plastic Coplin jar of Retrievit, to be warmed in microwave
    - **Microwave on MEDIUM**, **12 minutes.**
    - Refill slide jars with hot Retrievit solution 4-5 times (approximately **every 2 minutes**)

      (One 3-jar run requires approximately 500 ml of Retrievit solution)

    **If running 10 slides, split slides into two 5-slide microwave runs; when the first is done, allow it to cool for the necessary 20 minutes, then place in PBS while the other half is finishing
8. Remove coplin jars, refill with hot solution; allow to cool with lid on loosely
    - **20 min.** @ RT
9. Rinse in dH2O
    - **2 min.**
    - **2 min.**
    - **2 min.**
10. Rinse in PBS

- **5 min.**

11. Circle sections with PAP pen (put other slides in PBS while working on circling, if necessary). Keep tissues moist. **If the tissue looks white/opaque, it is DRY—and this is bad!
12. Incubate with **Avidin D** solution **15 minutes**
13. Rinse briefly with PBS (dip slide in PBS—in and out!)
14. Incubate with **Biotin** solution **15 minutes**
15. During above incubation, prep **Blocking Solution** (10% Normal Rabbit Serum, Jackson, Cat. # 011-000-120); keep on ice

    <u>200 µl N.R.S. + 1800 µl PBS</u>
16. Rinse briefly with PBS (dip slide in PBS—in and out!)
17. Immediately treat slides with 60 µl **10% Normal Rabbit Serum**

    - **20 min.** @ RT in humid chamber (No MORE than 30 minutes—max)
18. Near end of the above 20 min. incubation, remove 1 eppitube of primary antibody (p21 WAF1 mouse IgG1 mAb) from 4º C refrigerator. Immediately before treating slides, dilute with sterile 1% Rabbit Serum

    19 µl N.R.S. + 1881 µl PBS = 1% Rabbit Serum

    <u>115 µl primary ab. + 1150 µl 1% Rabbit Serum</u>
19. Treat slides with 60 ml of dilute primary antibody per section

    <u>Incubate overnight</u> @ 4º C in humidified chamber

    *Negative control: no primary antibody—use 1% rabbit serum**

✯✯ 17 Hours Later:

20. Take DAB out of freezer and put in drawer to thaw

21. Wash in PBS

    - **5 min.**
    - **5 min.**
    - **5 min.**

22. During above washes, prep secondary antibody dilution (Biotinylated rabbit anti-mouse, DAKO, Cat. # E 0354)

    19 µl N.R.S. + 1881 µl PBS = 1% Rabbit Serum

    <u>7.6 µl secondary ab. + 1900 µl 1% Rabbit Serum</u>

    - Incubate **1 hr.** @ RT in humidified chamber (in the dark)

23. *During last fifteen minutes of above 1hr. incubation*, prep the **A and B reagents of Vectastain Elite ABC Kit**

    - Add **5 ml** PBS, **2 drops** from bottle A, swirl, **2 drops** from bottle B, and swirl well in larger gray transfer bottle
    - Set in dark drawer @ RT for **30 min.**

24. Wash slides in PBS

    - **5 min.**
    - **5 min.**
    - **5 min.**

25. Apply Vectastain Elite reagent

    - **30 min.** @ RT in humidified chamber

26. *During above 30 min. incubation*, prepare DAB

- **Filter DAB** (50 mg/ml) through PBS-dampened Whatman #1 filter paper 250 ml glass boat, use **2.0 ml** DAB
- Follow with PBS through filter 250 ml glass boat, use **198 ml** PBS (total = 200 ml)
- **IMMEDIATELY before staining, add 30% H2O2** 250 ml glass boat, use **100 µl** H2O2

27. Apply DAB

Immerse slides, stain until light-brown color appears **(20 sec.)**

28. Rinse in dH2O

- **1 min.**
- **1 min.**
- **1 min.**

29. During the above rinses, deactivate DAB—place DAB vile and equipment in bleach bucket; leave overnight

30. Dehydrate

- 70% EtOH, **1 min.**
- 95% EtOH, **1 min.**
- 100% EtOH, **1 min.**
- Xylene, **2 min.**

31. Mount with Permount and coverslip.

Product Sources:

Xylene: X3P, Fisher, # UN1307

PAP pen: Super HT, Research Products International Corp., # 195506

PBS: Life Technology, # 21600-069

Hydrogen Peroxide: Sigma, # H-1009

Retrievit Target Retrieval Solution: InnoGenex, # BS-1002-00

Normal Rabbit Serum: Jackson, # 011-000-120

Avidin/Biotin Blocking Kit: Vector, # SP-2001

Primary antibody, purified mouse Anti-p21 mAb: SXM30, BD Biosciences Pharmingen, # 556431

Secondary antibody, biotinylated rabbit anti-mouse: DAKO, # E0354

ABC Reagent: Vectastain Elite Kit, Vector Lab, # PK-6102

DAB: Diaminobenzidine-tetrahydrochloride, Sigma, # D5637

Whatman #1 filter paper: Whatman, # 1001 185

Permount: Fisher, # SP15-100

References:

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Viale, Giuseppe, Caterina Pellegrini, Giovanni Mazzarol, Patrick Maisonneuve, Mark L. Silverman, and Silvano Bosari. p21$^{WAF1/CIP1}$ expression in colorectal carcinoma correlates with advanced disease state and p53 mutations. *Journal of Pathology* 1999, 187: 302-307.

Ruan, Sanbao, Greg Fuller, Victor Levin, Janet M. Bruner, and Wei Zhang. Detection of p21$^{WAF1/Cip1}$ in brain metastases. *Journal of Neuro-Oncology* 1998, 37: 223-228.

APPENDIX G

HISTONE H4 ACETYLATION DETECTION ASSAY PROTOCOL

(Kristy L. Covert, 3/2004)

For use with 4 micron sections of 4% PFA-fixed, paraffin-embedded rat colon tissue

Run Info

Date: _____

Start Time: _____
Time Into 4º: _____
Time Out of 4º: _____
Finish Time: _____

Slide #1: _____
Slide #2: _____
Slide #3: _____
Slide #4: _____
Slide #5: _____
Slide #6: _____
Slide #7: _____
Slide #8: _____
Slide #9: _____

1. Deparaffinize slides in xylene
   - ❑ **5 min.**
   - ❑ **5 min.**
   - ❑ **5 min.**
2. Rehydrate in graded alcohols
   - ❑ 100%, **2 min.**
   - ❑ 100%, **2 min.**
   - ❑ 95%, **2 min.**
   - ❑ 95%, **2 min.**
   - ❑ 70%, **2 min.**
3. Wash in PBS, **5 min.**
4. Quench endogenous peroxidase

   250 ml glass boat, add **<u>180 ml methanol + 20 ml 30% H2O2</u>**, for 200 ml total dilute solution—add H2O2 immediately before using

   - ❑ 3% H2O2, **30 min.** @ RT
5. Rinse in PBS
   - ❑ **5 min.**

- **5 min.**

6. During above second rinse, warm diluted Retrievit antigen retrieval solution
    - **3 min. on HIGH (or until it comes to rolling boil)**
7. Antigen Retrieval
    - Place 3 slides (per plastic Coplin jar) in warm Retrievit solution (add a blank slide, if necessary)
    - Include one extra plastic Coplin jar of Retrievit, to be warmed in microwave
    - **Microwave on MEDIUM**, **12 minutes.**
    - Refill slide jars with hot Retrievit solution 4-5 times (approximately **every 2 minutes**)

      (One 3-jar run requires approximately 500 ml of Retrievit solution)

    **If running 10 slides, split slides into two 5-slide microwave runs; when the first is done, allow it to cool for the necessary 20 minutes, then place in PBS while the other half is finishing
8. Remove coplin jars, refill with hot solution; allow to cool
    - **20 min.** @ RT
9. Rinse in dH2O
    - **2 min.**
    - **2 min.**
    - **2 min.**
10. Rinse in PBS

- **5 min.**

11. Circle sections with PAP pen (put other slides in PBS while working on circling, if necessary). Keep tissues moist. **If the tissue looks white/opaque, it is DRY—and this is bad!
12. Incubate with **Avidin D** solution **15 minutes**
13. Rinse briefly with PBS (dip slide in PBS—in and out!)
14. Incubate with **Biotin** solution **15 minutes**
15. Rinse briefly with PBS (dip slide in PBS—in and out!)
16. During step #14 incubation, prep **Blocking Solution** (10% Normal Goat Serum, Jackson, Cat. # 005-000-121); keep on ice

    180 µl N.G.S. + 1620 µl PBS = 1800 µl 10% Normal Goat Serum
17. Incubate slides with 60 µl **10% Normal Goat Serum**
    - **20 min.** @ RT in humid chamber (No MORE than 30 minutes—max)
18. Near end of the above 20 min. incubation, remove 1 eppitube of primary antibody (RABBIT POLYCLONAL ANTI-ACETYL HISTONE H4, UPSTATE, #06-598) from 4º refrigerator. Immediately before treating slides, dilute with sterile 1% Goat Serum

    24 µl N.G.S. + 2376 µl PBS = 2400 µl 1% Goat Serum

    Then, **take out 1500 µl** 1% Goat Serum for Primary Antibody

    (Remainder is for Negative)

    1:750 = 2 µl primary ab. + 1500 µl 1% Goat Serum
19. *Do not rinse* slides before applying Primary Antibody!!

Treat slides with 60 ml of dilute primary antibody per section—**1:1750 dilution**.

Incubate overnight @ 4º C in humidified chamber

Negative control: no primary antibody—use 1% goat serum only

✯✯ 24 Hours From Start of Yesterday's Assay:

20. Take DAB out of freezer and put in drawer to thaw
21. Wash in PBS
    - **5 min.**
    - **5 min.**
    - **5 min.**
22. During above washes, prep secondary antibody dilution (JACKSON BIOTIN-SP-AFFINIPURE GOAT ANTI-RABBIT IgG (H+L), #111-065-144), **1:200 dilution**.

    18 µl N.G.S. + 1782 µl PBS = 1800 µl of 1% Goat Serum

    1:200 = 9 µl secondary ab. + 1800 µl 1% Goat Serum

    - Incubate **1 hr.** @ RT in humidified chamber (in the dark)
23. *During last fifteen minutes of above 1hr. incubation*, prep the **A and B reagents of Vectastain Elite Standard ABC Kit (#PK-6102)**
    - Add **5 ml** PBS, **2 drops** from bottle A, swirl, **2 drops** from bottle B, and swirl well in larger gray transfer bottle
    - Set in dark drawer @ RT for **30 min.**
24. Wash slides in PBS
    - **5 min.**

- **5 min.**
- **5 min.**

25. Apply Vectastain Elite reagent
    - **30 min.** @ RT in humidified chamber
26. *During above 30 min. incubation*, prepare DAB
    - **Filter DAB** (50 mg/ml) through PBS-dampened Whatman #1 filter paper

      250 ml glass boat, use **2.0 ml** DAB
    - Follow with PBS through filter

      250 ml glass boat, use **198 ml** PBS (total = 200 ml)
    - **IMMEDIATELY before staining, add 30% H2O2**

      250 ml glass boat, use **100 µl** H2O2
27. Apply DAB

    Immerse slides, stain until light-brown color appears **(20 sec.)**
28. Rinse in dH2O
    - **1 min.**
    - **1 min.**
    - **1 min.**
29. During the above rinses, deactivate DAB—place DAB vile and equipment in bleach bucket; leave overnight
30. Dehydrate
    - 70% EtOH, **1 min.**

- 95% EtOH, **1 min.**
- 100% EtOH, **1 min.**
- Xylene, **2 min.**
- Xylene, **2 min.**

31. Mount with Permount and coverslip.

Product Sources:

Xylene: X3P, Fisher, # UN1307

PAP pen: Super HT, Research Products International Corp., # 195506

PBS: Life Technology, # 21600-069

Hydrogen Peroxide: Sigma, # H-1009

Retrievit Target Retrieval Solution: InnoGenex, # BS-1002-00

Normal Goat Serum: Jackson, # 005-000-121

Avidin/Biotin Blocking Kit: Vector, # SP-2001

Primary antibody, Anti-acetyl-Histone H4 (rabbit polyclonal IgG): Upstate, # 06-598

Secondary antibody, Biotin-SP-AffiniPure Goat Anti-Rabbit IgG (H+L): Jackson, # 111-065-144

ABC Reagent: Vectastain Elite Standard Kit, Vector Lab, # PK-6102

DAB: Diaminobenzidine-tetrahydrochloride, Sigma, # D5637

Whatman #1 filter paper: Whatman, # 1001 185

Permount: Fisher, # SP15-100

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