COLON CANCER CHEMOPROTECTION THROUGH EPIGENETIC
EFFECTS OF A FISH OIL/PECTIN DIET

A Dissertation

by

YOUNG MI CHO

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

DOCTOR OF PHILOSOPHY

August 2012

Major Subject: Nutrition
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Accumulated genetic and epigenetic abnormalities contribute to the development of colon cancer. We have shown that a combination of fish oil (containing decosahexaenoic acid, DHA, 22:6 n-3) and pectin (fermented to butyrate by colonic microflora) is protective against colon carcinogenesis in part by regulating the expression of genes involved in apoptosis, leading to apoptosis induction.

To determine how FO/P enhances apoptosis, we measured the expression of genes involved in apoptosis. We performed a pathway analysis on differentially expressed genes identified at three times during colon tumorigenesis: initiation, aberrant crypt foci (ACF) formation, and tumor stage, and compared these results with phenotypic observations at those times. At initiation, FO/P down-regulated the expression of genes involved with cell adhesion and enhanced apoptosis compared with corn oil/cellulose (CO/C). At the ACF stage, expression of genes involved in cell cycle regulation was modulated by FO/P and proliferation was reduced in FO/P rats compared
with CO/C rats. FO/P increased apoptosis and the expression of genes that promote apoptosis at the tumor endpoint compared with CO/C.

We next determined if changes in expression of genes involved in apoptosis by FO/P are associated with changes in promoter methylation of a key apoptosis regulator, \textit{Bcl-2}. Genomic DNA was isolated from carcinogen-induced colon tumors and non-involved tissues. FO/P increased \textit{Bcl-2} promoter methylation in tumor tissues and colonocyte apoptosis relative to those observed with CO/C. A negative correlation between \textit{Bcl-2} DNA methylation and \textit{Bcl-2} mRNA levels was observed in the tumors.

Additionally, we examined gene specific promoter methylation of 24 apoptosis-related genes using human colon cancer cells. Cells were treated with DHA or linoleic acid (18:2 n-6), and select cultures were also treated with butyrate. The combination of DHA and butyrate led to a significant reduction in the methylation of pro-apoptotic genes and an increase in apoptosis. These data suggest that part of the mechanisms involved in the induction of apoptosis by FO/P may be through epigenetic regulation of genes involved in apoptosis throughout colon carcinogenesis.
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CHAPTER I
INTRODUCTION AND LITERATURE REVIEW

Colon cancer development

Introduction

Colon cancer is the second leading cause of cancer mortality in the United States (1). In 2010, estimated deaths for colon cancer were 51,370, and 102,900 people were newly diagnosed with colon cancer in the United States. Moreover, colon cancer incidence among Asians who migrated to the United States was higher than reported among those living in their homeland, suggesting that environmental factors such as diet and physical activity contribute to colon cancer incidence (2). It has been estimated that up to 80% of colon cancers may be preventable by dietary intervention (3).

Colon physiology

The functions of the colon include absorption of sodium, chloride, and water as well as the secretion of potassium and bicarbonate. The colon epithelia also functions as a barrier from fecal material (4). The colon epithelium is organized into crypts, which increases the colon surface area in order to enhance absorption. The colon contains microflora, which can digest and ferment materials, such as fiber, that are unable to be digested by humans. Products of microbial fermentation include vitamins and short chain fatty acids (e.g., acetate, propionate, and butyrate), which can then be absorbed for

This dissertation follows the style and format of the Journal of Nutrition.
utilization by the body or the colon cells (5).

The colon epithelium is a dynamic system that is dependent upon a balance of cell birth, differentiation, and apoptosis (programmed cell death) to maintain tissue homeostasis. Colon epithelial cells are derived from stem cells in the bottom of the crypt and migrate along the crypt column toward the mucosal surface. Cells differentiate into their mature phenotypes in the middle and upper portion of the crypt, and typically only undergo apoptosis in the upper part of the crypt. Apoptotic and necrotic, colonocytes are found in the fecal stream, along with live cells eliminated by sloughing.

Colonocytes have a very rapid turnover, with all cells being replaced within 3-6 days in humans (6) and 2-4 days in rodents (7). Due to the rapid turnover of colon epithelial cells, the colon is a common site for abnormal growths, such as adenomas and carcinomas (8).

**Apoptosis and colon cancer**

In the colon crypts, epithelial cells are rapidly renewing but the cell population is maintained in a steady state by the balance of cell proliferation and cell loss. If this balance is disturbed and the rate of cell proliferation surpasses that of cell sloughing and apoptosis, cells can accumulate, and if they are transformed, they can develop into a tumor (9). Apoptosis plays a critical role in sustaining cell homeostasis by allowing the removal of damaged cells. Apoptosis is regulated by two major pathways; 1) the extrinsic death receptor pathway, and/or 2) the intrinsic mitochondrial pathway. In both pathways, cysteine aspartyl-specific proteases (caspases) are activated leading to cleavage of cellular substrates and morphological changes. The morphological changes
in apoptotic cells are nuclear DNA fragmentation, cell shrinkage and membrane
blebbing (10). The extrinsic pathway is triggered by the binding of ligands to death
receptors on the cell membrane; 1) the Fas ligand to the Fas (CD95/APO-1) receptor, 2)
the tumor necrosis factor (TNF) ligand to the TNF receptor, or 3) the TNF-related
apoptosis inducing ligand (TRAIL) to the DR4 and DR5 receptors. Each receptor has an
intracellular domain that recruits adaptor molecules, resulting in activation of caspases
(11).

The intrinsic pathway occurs in the mitochondrial membrane with cytochrome C
release. In the cytosol, released cytochrome C forms an apoptosome with apoptotic
protease activating factor 1 (Apaf-1) and caspase 9, resulting in apoptosis (12). Over-
expression of Bcl-2 inhibits apoptosis and is found in various cancers, including colon
cancer (13). Members of the Bcl-2 family are mainly located at the mitochondrial
membrane, and include both anti- and pro-apoptotic proteins. Anti-apoptotic members
are Bcl-2, Bcl-X\textsubscript{L}, Bcl-w, and MCL1, and they contain the Bcl-2 homology (BH)
domains 1, 2, and 4. Pro-apoptotic members are subdivided into a Bax subfamily (Bax,
Bak, and Bok) and BH3-only proteins (Bid, Bad, Bik, Hrk, and Bim). Pro-apoptotic
effects of BH3-only proteins bind and inactivate anti-apoptotic proteins. Bax and Bak
are known to undergo conformational changes prior to their insertion into the outer
mitochondrial membrane, thereby creating channels that allow cytochrome C release.
Bcl-2 is able to inhibit the conformational changes and/or oligomerization of Bax and
Bak within the mitochondrial membrane, thereby inhibiting apoptosis (14). p53 has a
vital role to induce apoptosis regarding the induction of pro-apoptotic genes, including Bax, Nova, and CD95 upon DNA damage (15).

**Genetic mutation in colon cancer**

DNA can be damaged not only by environmental factors such as UV light and radiation but also by reactive oxygen species generated by metabolism. DNA surveillance mechanisms repair up to one million DNA damage sites per day. Failure in DNA damage repair contributes to accumulation of damage enhancing the probability of gene mutations (16). Mutations of the DNA sequence disrupt the expression and/or function of genes. The changes in sequence and their subsequent effects are not reversible. Genetic etiology of colon cancer is well established. In 1990, Fearon and Vogelstein proposed the multi-step development of colon tumorigenesis (17).

Mutation of oncogenes mainly causes gain-of-function through transcriptional or post-transcriptional modifications in the gene products. For example, mutation of *BRAF* in human cancers activates BRAF kinase, resulting in phosphorylation of downstream targets such as Mitogen activated protein kinase (MAPK), which leads to aberrant growth of cells (18). In contrast, mutations that occur in tumor suppressor genes are often loss-of-function mutations that reduce the activity of the gene product. Mutational inactivation of tumor suppressor genes in colon carcinogenesis occurs in *Apc* and *Tp53*. At the initiation stage of carcinogenesis, *Apc* is mutated in 85% of sporadic colorectal cancers (19). This mutation causes activation of the Wnt signaling pathway by inhibiting degradation of β-catenin oncoprotein (20, 21). Mutations of *Tp53* inactivates the transcriptional activity of p53, which normally mediates cell-cycle arrest and a cell-death
checkpoint (22). Accumulated mutations of oncogenes and tumor suppressor genes directly disturb cell homeostasis by activating genes involved in cell proliferation and by inhibiting pro-apoptotic genes.

**Non-invasive colon cancer detection**

Ninety percent of patients survive if colon adenomas are detected at an early stage of malignant transformation (23). Therefore, early detection of initial changes in the colon is essential for the reduction of colon cancer death rate. The rapid turnover rate of colon epithelial cells (3-6 days in humans) makes it feasible to utilize exfoliated colonocytes in fecal material as a non-invasive technique to detect changes in gene expression.

Genetic and epigenetic alterations in fecal DNA have been tested as a non-invasive screening tool. Traverso et al. reported that mutations of the *APC* gene were able to be detected using fecal DNA from colorectal cancer patients. (24) The type of microRNA from human stool was tested as a non-invasive screening tool, and it showed a unique and identifiable pattern in colorectal neoplasms (25). The study by Chen et al. demonstrated that the promoter region of *Vimentin* was methylated in colon tumors and was detectable in fecal DNA from colon cancer patients but not in normal colon tissues (26). Another group found aberrantly methylated *CDKN2A, MGMT, and MLH1* in colon polyps and in fecal DNA from patients with colorectal polyps (27). Glockner et al. demonstrated that *TFPI2* methylation may serve as a biomarker for the early detection of colon cancer using stool DNA in patients with colon cancer (28).
Many trials have been conducted in order to develop a non-invasive technique to detect alterations in gene expression using mRNA isolated from exfoliated colonocytes. Isolated poly A+ RNA from fecal samples revealed that PKC βII expression was 2.5-fold higher in carcinogen injected rats compared to saline injected rats (29). In a separate study, human subjects with colon adenomas exhibited higher mRNA level of cyclin D1, and subjects with colon inflammation had elevated levels of COX-2 (30). Data from these experiments support the conclusion that mRNA isolated from exfoliated colonocytes could be used to monitor changes in gene expression during colon carcinogenesis. Therefore, the non-invasive technique using exfoliated colonocytes makes it feasible to evaluate the effects of a chemopreventive diet on all stages of colon cancer within the same subject.

**Epigenetic modification in chromatin structure**

**Introduction**

Epigenetics refers to the study of changes in phenotype or gene expression derived from mechanisms other than DNA sequence changes (31). Epigenetic regulation of gene expression is critical to organism development and cellular differentiation, but it is also a key element in the development of cancer (32). During carcinogenesis, gene transcription is aberrantly regulated via epigenetic changes of chromatin structure. These changes include DNA methylation and histone modification (i.e. acetylation,
methylation, and phosphorylation), and these modifications occur primarily in the promoter region of genes (33, 34).

**DNA methylation**

DNA methylation occurs primarily at cytosine-guanosine dinucleotides (CpG) by the addition of a methyl group at the 5’ position of cytosine. CpG islands are CpG-rich regions in or near the promoter region of genes (35). DNA methylation is mediated by DNA methyltransferases, which transfer methyl groups from S-adenosyl methionine to cytosines within CpG dinucleotides. DNA methyltransferase 1 (DNMT1) is known to maintain DNA methylation by catalyzing the addition of a methyl group to newly synthesized strands of hemi-methylated DNA, whereas DNMT3A and 3B are de novo methyltransferases required for initiating and establishing new methylation during development.

Mechanisms whereby DNA methylation hinders transcription have been proposed. Perini et al. demonstrated that methylation within the promoter region of N-Myc target genes prevents transcription factor binding to those sequences (36). Methylated DNA provides a binding site for methyl-binding proteins (MBPs), which form a repressive complex with histone deacetylases (HDAC) and histone methyltransferases (HMT) (37). MBPs act as important translators between DNA methylation and histone modifier genes that contribute a transcriptional silencing of genes by chromatin remodeling (Figure 1).
Figure 1. Epigenetic regulation by DNA methyltransferases, methyl-binding proteins, and histone modifying enzymes. DNA is methylated by DNA methyltransferases (DNMTs). Methylated cytosine residues (solid circles) are bound by methyl-binding proteins (MBPs) that subsequently recruit histone deacetylases (HDACs) and histone methyltransferases (HMTs). These enzymes mediate complex changes in the histone modification pattern of methylated genes that result in the establishment of repressive chromatin structures. acH4K12 = lysine 12 – acetylated histone H4; acH4K5 = lysine 5 – acetylated histone H4; mH3K9 = lysine 9 – methylated histone H3; mono, di, tri = mono-, di-, tri-methylated; open circles = unmethylated cytosine residues. Image was reprinted with permission from “DNA Methyltransferase Inhibitors and the Development of Epigenetic Cancer Therapies (38)” Copyright 2005 by Oxford University Press.

Cancer cells exhibit aberrant DNA methylation patterns, global hypomethylation and gene-specific promoter hypermethylation of tumor suppressor genes. Global hypomethylation often occurs in cancers, particularly within regions of the genome containing extensive amounts of repetitive elements and retrotransposons (39). Chromosomal instability due to global hypomethylation might induce tumor initiation (40). However, more attention has been focused on aberrant epigenetic silencing of tumor suppressor genes by promoter hypermethylation. According to a recent review
regarding DNA methylation and colon cancer, accumulated studies have revealed that 59 genes were highly methylated in biopsy, stool, and serum samples of colon cancer patients (41). For instance, *CDKN2A/p16* and *MGMT*, which are tumor suppressor genes involved in cell cycle control and DNA repair, were silenced by DNA methylation in colon carcinogenesis (27, 42). Data indicate promoter hypermethylation of genes involved in apoptosis suppresses the transcription levels of those genes, resulting in the inhibition of apoptosis in cancerous cells (43). For instance, *Dapk1* is a positive mediator of apoptosis, and thus a tumor suppressor candidate, and it is heavily methylated in colon and bladder cancer (44, 45).

In contrast to hypermethylation of tumor suppressor genes, hypomethylation of tumor promoters can facilitate tumorigenesis. For example, a region in the first exon of the *Bcl-2* oncogene is completely demethylated allowing high-levels of *Bcl-2* expression in B-cell chronic lymphocytic leukemia and colon cancer (46, 47).

Because regulation of promoter methylation is apparently a contributing factor in colon carcinogenesis, it is important to study potential methods of reestablishing normal methylation patterns. Using animal models, it would be possible to study this process *in vivo* but using controlled conditions, such as diet. For example, Borinstein et al. assessed DNA methylation in AOM-induced colon cancer and found cancer-specific aberrant DNA methylation of *Zik1* and *Gja9*, whereas *p16 Ink4a, Igfbp3, Mgmt, Id4*, and *Cxcr4* were methylated in both the AOM-induced tumors and normal colon mucosa (48). However, there are few studies that have used this method to determine the effect of diet on DNA methylation.
Histone modification

Histone acetylation facilitates the binding of transcription factors to promoters and disrupts higher order chromosome structure, promoting transcription. Jenuwein and Allis proposed the “histone code” which suggests that histone protein modification contributes to the alteration of chromatin structure, that leads to inherited differences in gene expression (49).

In addition to DNA methylation, histone modification is another epigenetic mechanism contributing to the regulation of gene transcription (50). Aberrant changes in histone modifications are found during cancer development (51). The nucleosome, consisting of DNA and an octomer of two each of histones H3, H4, H2A and H2B is the unit of chromatin (52). Chromatin structure is flexible, and the changes in chromatin structure caused by chemical modifications to the lysine on histone amino terminal tails contribute to the regulation of gene transcription.

Histone acetylation depends on the balance between the activities of histone acetyltransferase (HAT) and histone deacetylase (HDAC) (53). Mutations or translocations of HAT genes increase susceptibility of cancer. Missense or truncating mutations of p300, which is one of the HAT genes, were found in human colon cancers (54). HDAC is a transcriptional repressor of tumor suppressor genes like p53 and RB and is highly expressed in cancers (55). Agents that inhibit HDACs, for instance butyrate, lead to maintenance of histone H4, H2B and H3 in the hyperacetylated state and promote the transcription of a variety of genes (56, 57).
In addition to histone acetylation, histone H3 and H4 are methylated at the lysine residue, resulting in changes in chromatin structure. Histone methyltransferase (HMT) catalyzes the addition of up to three methyl groups to lysine residues. Methylation of H3K4, H3K36, and H3K79 induces a chromatin structure that promotes gene expression, whereas that of H3K9, H3K27, and H4K20 induces a chromatin structure that inhibits expression (58).

Another chromatin modifier, polycomb group (PcG) and trithorax group (TrxG) proteins function coordinately to modulate DNA accessibility throughout neonatal development (59). The PcG complex represses gene expression by regulation of H3K27 methylation, whereas TrxG proteins promote transcription through regulation of H3K4 (60, 61). PcG complex has been proposed to be involved in cell cycle regulation and stem cell biology. Also PcG proteins bind to noncoding RNAs and components of RNA interference, mediating transcription gene silencing (62).

**Demethylating agents**

In contrast to genetic mutations, epigenetic changes can be reversed. Restoration of tumor suppressor transcription by the reversal of these epigenetic processes is considered as a potential modality for cancer therapeutics. The DNA methyltransferase inhibitor 5-aza-2-deoxycytidine (5-Aza-dC, i.e., decitabine) has been approved by the Food and Drug Administration as an antitumor agent for the treatment of hematological malignancies, such as myelodysplastic syndromes (63) that exhibit aberrant DNA methylation patterns (64).
5-Aza-dC is directly incorporated into DNA and acts as a substrate for DNMT. However, it adheres to DNMT and remains as a covalent protein-DNA adduct, thereby suppressing DNA methylation (65). Kantarjian et al. demonstrated 69% of patients treated with decitabine experienced severe side effects including neutropenia, thrombocytopenia, febrile neutropenia, leucopenia, pyrexia, hyperbilirubinemia, and pneumonia (66). In order to avoid the side effects caused by this pharmacological approach, attempts are being made to find natural dietary compounds that exert demethylating effects.

Epigallocatechin-3-gallate (EGCG, green tea compound) interrupts DNMT activity by blocking the active site of the enzyme (67). Nandakumar et al. demonstrated that green tea polyphenols restore the expression of the tumor suppressor genes Cip1/p21 and p16$^{INK4a}$ in human skin cancer cells by suppressing DNA methylation (68). Using the AOM-treated Apc$^{Min/+}$ mouse model, Volate et al. determined that consumption of low concentrations of green tea led to demethylation of the RXRα promoter region, which restored RXRα expression in colon mucosa (69).

Fang et al. reported that genistein, a soy isoflavone, reversed hypermethylation and reactivated RARβ, p16$^{INK4a}$ and Mgmt expression in human esophageal squamous cancer cells (70). Shu et al. found that curcumin resulted in a 29% reduction of promoter methylation of Neurog1 in prostate cancer cells, and this reduction led to the 2-fold increase of Neurog1 mRNA expression (71). Fini et al. revealed that Annurca apple polyphenols reduced DNA methylation of the promoters of hMLH1, p14$^{ARF}$, and p16$^{INK4a}$ and restored those genes’ expression by reduction of DNMT proteins (72).
The supply of methyl group in one-carbon metabolism

Dietary factors including folate, vitamin B12, B6, B2, methionine, and choline influence the supply of methyl groups in one-carbon metabolism (Figure 2). These nutrients play an essential role as cofactors for biosynthesis of S-adenosyl methionine which is the methyl donor for methylation reactions (73).

Dietary folate is converted to 5 methyltetrahydrofolate (5-MTHF) by the enzyme methylene tetrahydrofolate reductase (MTHFR). A carbon unit from serine or glycine is transferred to tetrahydrofolate (THF) to form 5,10-MTHF. The transfer of the methyl group from 5-MTHF to homocysteine requires vitamin B12 and results in the synthesis of methionine, which is converted into S-adenosyl methionine.

There have been inconsistent data pertaining to the effect of folate deficiency on DNA methylation. Cravo et al. proposed that a mechanism by which folate deficiency enhances colon carcinogenesis might be through an induction of genomic DNA hypomethylation (74). However, Balaghi and Wagner demonstrated that a prolonged folate deficiency failed to induce genomic DNA hypomethylation in liver even though it significantly reduced hepatic S-adenosyl methionine levels (75). The supplementation of folate to subjects with colon adenomas increased the extent of genomic DNA methylation at 6 months and 1 year but placebo administration increased it at 1 year, suggesting that the timing and duration of folate intervention is important in terms of DNA methylation (76).
Figure 2. One-carbon metabolic cycle. Nutrients related to one carbon metabolism contribute to the status of DNA methylation through the modulation of methyl group supply. B2, vitamin B2; B6, vitamin B6; B12, vitamin B12.

DHA may also affect DNA methylation because incorporation of DHA into membrane phospholipids requires S-adenosyl methionine-derived methyl group utilization to support phospholipid metabolism. Methyl groups are transferred by phosphatidyl ethanolamine-N-methyltransferase (PEMT) to ethanolamine in a series of steps that convert it to phosphatidylcholine. A depletion of DHA in membrane phospholipids caused by low dietary DHA levels would reduce the demand for methyl groups to support phosphatidylcholine synthesis, and thus may increase availability of methyl groups for histone and DNA methylation (77).
Chemoprotection of dietary fish oil and pectin against colon cancer

**Fish oil rich in n-3 PUFA**

Fish oil is rich in n-3 PUFA such as eicosapentaenoic acid (20:5\(\Delta^{5,8,11,14,17}\)) and decosahexaenoic acid (22:6\(\Delta^{4,7,10,13,16,19}\)). In contrast, vegetable oils are known to contain high levels of n-6 PUFA, such as linoleic acid (18:2\(\Delta^{9,12}\)) and arachidonic acid (20:4\(\Delta^{5,8,11,14}\)). n-3 PUFAs in fish oil are incorporated into the phospholipids of the mitochondrial membrane (78). For instance, DHA is primarily incorporated into phosphatidylethanolamine (PE) which can be converted to phosphatidylcholine (PC) by adding a methyl group from S-adenosyl methionine (79). Because n-3 PUFA is highly unsaturated, they enhance susceptibility to damage by reactive oxygen species generated via oxidative phosphorylation (80). Peroxidation of phospholipids induces the release of cytochrome C from mitochondria into the cytosol, thereby facilitating induction of apoptosis (81).

We have demonstrated that n-3 PUFA promotes colonocyte apoptosis and reduces colon tumor formation in part by suppressing oncogenic Ras activation and NF-κB signaling in colonocytes (82). Another mechanism of chemoprotection by fish oil is to induce apoptosis for removal of DNA damaged cells during colon cancer initiation (83) and spontaneous apoptosis during colon cancer progression (84, 85). Fish oil enhances apoptosis in part by suppression of Bcl-2 levels, an anti-apoptotic mediator (86).
Microarray analysis to compare global changes in gene expression profiles in AOM-injected rats as a function of dietary lipid (fish oil, corn oil, or olive oil) revealed that dietary fatty acid composition alters gene expression profiles in the colonic epithelium at both the initiation and promotional stages of tumor development (84). Therefore, fish oil or n-3 PUFA are capable of influencing gene expression throughout colon carcinogenesis.

**Butyrate from fermentable fiber, pectin**

Butyrate is a product of microbial fermentation of fermentable fibers, such as pectin, and is a major energy source of colonocytes. Butyrate is also a HDAC inhibitor and hydrophobic pockets of HDAC facilitate its interaction with butyrate. Two butyrate molecules are able to occupy the active site of HDAC, thereby inhibiting the enzyme (56).

Chemoprotective effects of butyrate include enhancement of apoptosis (87, 88) and suppression of proliferation (89, 90) in colon cancer cell lines. The underlying mechanisms by which butyrate contributes to the changes in apoptosis, and cell cycle arrest include the effect of butyrate on HDAC inhibition, which elicits the transcriptional regulation of genes involved in those processes. For instance, p21, the cyclin-dependent kinase inhibitor, was up-regulated by butyrate treatment in p53-null cells, and this increase was dependent on Sp1 response elements in the p21 promoter (91). A subsequent study reported that Sp1/Sp3 recruit HDACs to the p21 promoter, resulting in histone deacetylation, whereas butyrate ameliorated the compressed chromatin structure, allowing the transcription of p21 and cell cycle arrest (56). Crim et al. reported that
butyrate, which was released from capsules in the colon of carcinogen-injected rats, elevated the expression of p21 by histone hyperacetylation \textit{in vivo} (92).

The inhibitory effect of butyrate on HDAC can lead to the induction of apoptosis. Nakata et al. demonstrated butyrate increased the expression of the death receptor 5/TRAIL-R2 and the consequent activation of caspases in human malignant tumor cells (93). Fan et al. also demonstrated that butyrate enhanced apoptosis through the Fas-mediated, extrinsic pathway in colonocytes (94).

\textbf{The combination of fish oil and pectin}

A study by Chang et al. demonstrated that the combination of FO/P is protective against colon carcinogenesis compared with CO/C, in part by enhancing colonocyte apoptosis (95). Studies from our lab continue to support that the induction of apoptosis contributes to chemoprotective effects of FO/P. Compelling evidence indicated that DHA and butyrate synergize to enhance mitochondrial Ca\(^{2+}\) accumulation, thereby inducing apoptosis (96, 97). With respect to another mechanism of action for enhancing apoptosis, dietary FO/P suppressed the levels of PPAR\(\delta\) and prostaglandin 2, and elevated prostaglandin 3 in both AOM-alone and irradiated AOM rats (98). We have recently demonstrated that FO/P also modulates non-coding microRNA in AOM-induced colon cancer, one of the epigenetic mechanisms whereby gene transcription is regulated (99). Although many aspects of the chemoprotective properties of FO/P have been explored, no study has investigated their effects on promoter methylation.
Summary and purpose

Colon cancer is the second leading cause of cancer mortality in the United States. Data suggests that environmental factors, such as diet, play a critical role in determining the risk of colon cancer. In addition to accumulated genetic mutations of tumor suppressors and promoter genes, aberrant epigenetic alterations have been proposed as a factor involved in colon carcinogenesis.

Dietary fat and fiber are the most widely investigated components in the chemoprevention of colon carcinogenesis. Reliable evidence emerging from studies using animal models and cells have shown that diets rich in n-3 PUFA found in fish oil, (rich in DHA and EPA) and pectin (a producer of butyrate) protect against colon carcinogenesis by enhancing cellular apoptosis. Although the chemoprotective properties of DHA and butyrate have been extensively investigated, their effects on epigenetic mechanisms have not been determined.

Therefore, this study aimed to further elucidate the chemoprotective properties of fish oil (DHA) and fermentable fiber or butyrate by determining their influence on gene expression. The goals included determining global changes in gene expression profiles at biologically important time points in carcinogen injected rats, and epigenetic regulation of genes involved in colonocyte apoptosis in vivo and in vitro. In an attempt to elucidate the mechanisms of action the following hypotheses and specific aims were designed:
**Hypotheses and specific aims**

1. The combination of FO/P compared to CO/C beneficially modulates gene expression during colon carcinogenesis.
   
   **Aim.** Determine gene expression using exfoliated colonocytes collected at three biologically important time points during colon tumorigenesis: initiation, ACF, and tumor stage.

2. Changes in phenotypes caused by FO/P compared with CO/C are due to changes in gene expression occurring during colon carcinogenesis.
   
   **Aim.** Compare fecal microarray results with phenotypic data (aberrant crypt foci, apoptosis, cell proliferation, and colon tumor incidence) to establish the regulatory controls contributing to the chemoprotective effects of FO/P.

3. Changes in expression of *Bcl-2* in response to a FO/P diet are associated with changes in methylation of the promoter region of *Bcl-2*, which is known to be hypomethylated in colon cancer.
   
   **Aim.** Determine the methylation status of specific CpG dinucleotides of the *Bcl-2* promoter region and the transcription level of *Bcl-2* in colon carcinomas.

4. DHA and/or butyrate enhance colonocyte apoptosis by affecting epigenetic regulation of apoptosis-related genes in colon cancer cells.
   
   **Aim.** Determine if n-3 PUFA and/or butyrate modulates the level of gene expression of apoptosis related genes by suppressing aberrant gene promoter methylation or enhancing histone acetylation.
CHAPTER II
A CHEMOPROTECTIVE FISH OIL- AND PECTIN-CONTAINING DIET
TEMPORALLY ALTERS GENE EXPRESSION PROFILES IN EXFOLIATED
RAT COLONOCYTES THROUGHOUT ONCOGENESIS*

We have demonstrated that fish oil- and pectin-containing (FO/P) diets protect against colon cancer compared with corn oil and cellulose (CO/C) by upregulating apoptosis and suppressing proliferation. To elucidate the mechanisms whereby FO/P diets induce apoptosis and suppress proliferation during the tumorigenic process, we analyzed the temporal gene expression profiles from exfoliated rat colonocytes. Rats consumed diets containing FO/P or CO/C and were injected with azoxymethane (AOM; 2 times, 15 mg/kg body weight, subcutaneously). Feces collected at initiation (24 h after AOM injection) and at aberrant crypt foci (ACF) (7 wk postinjection) and tumor (28 wk postinjection) stages of colon cancer were used for poly (A)+ RNA extraction. Gene expression signatures were determined using Codelink arrays. Changes in phenotypes (ACF, apoptosis, proliferation, and tumor incidence) were measured to establish the regulatory controls contributing to the chemoprotective effects of FO/P. At initiation, FO/P downregulated the expression of 3 genes involved with cell adhesion and enhanced apoptosis compared with CO/C. At the ACF stage, the expression of genes involved

in cell cycle regulation was modulated by FO/P and the zone of proliferation was reduced in FO/P rats compared with CO/C rats. FO/P also increased apoptosis and the expression of genes that promote apoptosis at the tumor endpoint compared with CO/C. We conclude that the effects of chemotherapeutic diets on epithelial cell gene expression can be monitored noninvasively throughout the tumorigenic process and that a FO/P diet is chemoprotective in part due to its ability to affect expression of genes involved in apoptosis and cell cycle regulation throughout all stages of tumorigenesis.

Introduction

Colon cancer continues to be the second highest contributor to cancer deaths in the United States (100). It has been estimated that up to 80% of colon cancers may be preventable by dietary intervention (3). We have demonstrated that diets containing the combination of fish oil and pectin (FO/P), result in a lower tumor incidence than diets containing corn oil and cellulose (CO/C) (101). FO is high in (n-3) fatty acids whereas corn oil is high in (n-6) fatty acids. Pectin is a highly fermentable fiber that yields more butyrate upon microbial fermentation whereas cellulose is poorly fermented. One of the mechanisms by which FO/P is protective against colon cancer is the induction of apoptosis, a programmed cell death that allows the removal of damaged cells (83, 98, 101). Tumor development depends not only on suppression of apoptosis, but also on an increase in cell proliferation. We have reported that a FO/P diet also suppresses cell proliferation relative to a CO/C diet (95, 101).
Cell cycle progression is mediated by cyclin dependent kinases (CDK) and cyclins which are under both transcriptional and post-transcriptional regulation (102). We have demonstrated that fish oil with butyrate increases the expression of $p21^{Waf1/Cip1}$, a CDK inhibitor (92). Apoptosis is regulated by multiple routes including extrinsic and intrinsic pathways as well as the integrins, which control cellular adhesion (103, 104). We have examined the effect of diet on apoptosis at various time points during carcinogenesis (83, 84, 98) and have reported that the expression of $bcl-2$, one of the anti-apoptotic factors in the intrinsic pathway, is down-regulated in colon cells of rats fed a fish oil-rich diet (86). Therefore, it is important to identify the regulatory relationships among genes during the tumorigenic process to further elucidate the synergistic chemoprotective effects of fermentable fiber and fish oil.

We have developed a noninvasive technique in which intact eukaryotic mRNA can be successfully isolated from exfoliated colonocytes to monitor gene expression profiles (29, 105-107). This novel technique facilitates the determination of changes in gene expression contributing to the regulation of apoptosis and cell proliferation during disease development. In this study, we are using this non-invasive methodology to monitor gene expression at three biologically important time points during colon tumorigenesis: initiation, aberrant crypt foci formation (ACF), and tumor stage. The fecal gene expression results were compared with phenotypic data at the same time points to determine the mechanisms underlying the chemoprotective effects of a FO/P diet.
Materials and methods

Animals and study

Male Sprague-Dawley rats (Harlan Teklad, Madison, WI) were used to study the chemoprotective effect of FO/P at the initiation, ACF, and tumor stages of colon cancer. The animal use protocol was approved by the Institutional Animal Care and Use Committee of Texas A&M University and conformed to National Institutes of Health (NIH) guidelines. Rats were housed individually in a temperature and humidity controlled animal facility with a 12 h light/dark cycle. After 1 wk of acclimation and 31 d receiving the experimental diets, rats were injected with azoxymethane (AOM; Sigma, St Louis, MO, 15 mg/kg body weight). For the initiation stage analyses, 22 rats were terminated 24 h after AOM injection. At termination, fecal material was collected and immediately homogenized in RNA isolation solution for microarray analysis and colon tissue samples were collected and processed as described below. Rats used for the ACF stage (n=40) were maintained using the same diet and treatment conditions with the exception that animals received a second AOM injection 1 wk after the first injection. Seven weeks after the second AOM injection, rats were terminated and colon tissue samples were collected. Rats for the tumor stage analyses (n=80) were raised using the same diet and treatment conditions as the ACF stage rats, except animals were terminated at 31 wk after the second AOM injection. Feces from the tumor stage rats were collected at 7 and 28 wk after the second AOM injection, and colon tissue samples were collected at termination.
**Diets**

Rats were assigned to receive a diet containing either FO/P or CO/C as previously described (101). All diets contained oils at 15% by weight and 30% of energy. The two lipid sources differed in fatty acid composition; FO contained higher amounts of eicosapentaenoic acid (EPA, 20:5 (n-3)) and docosahexanoic acid (DHA, 22:6 (n-3)) than CO, which had higher amounts of linoleic acid (LA, 18:2 (n-6)). The fish oil diet included 3.5 g corn oil/100 g diet to prevent essential fatty acid deficiency. The amount of fiber in the diet was 6% by weight, which is equivalent to 30 g/d for humans. Fiber sources had differences in fermentability; pectin is highly fermentable whereas cellulose is poorly fermented. Citrus pectin was obtained from Danisco Cultor (New Century, KS) and cellulose was provided from Harlan Teklad (Madison, WI). Corn oil and bulk vacuum-deodorized menhaden fish oil were obtained from Degussa (Waukesha, WI). The antioxidant levels in the diets were balanced by including 15 mg d-α-tocopherol, 14 mg d-γ-tocopherol, and 5 mg tertiary butylhydroquinone/100 g diet in the FO/P diet and 19 mg tertiary butylhydroquinone/100 g diet in the CO/C diet. Animals were provided with fresh diet daily to prevent lipid oxidation, and given *ad libitum* access to food and water.

**Tissue collection**

Rats were killed by CO₂ overdose and cervical dislocation. The colon was resected and 1 cm of the distal colon was fixed in 4% paraformaldehyde (PFA) and another 1 cm of distal colon was used for 70% ethanol fixation. At the ACF stage, the
remaining colon was used for ACF scoring. Tissues from the tumor stage were evaluated for tumor incidence.

**RNA isolation from fecal samples**

To enrich the level of eukaryotic mRNA in the fecal samples, poly (A)+ RNA was isolated from total RNA using oligo dT cellulose micro spin columns and the mTRAP Maxi kit (Active Motif, Carlsbad, CA) (105). Fecal poly (A)+ RNA isolation was followed by DNase treatment (Appendix B-2) and aliquots were analyzed on an Agilent Bioanalyzer 2100 (Agilent, Palo Alto, CA) to assess mRNA quality and quantity (Appendix B-1). The remaining sample was used for microarray analyses.

**Microarray data acquisition**

Fecal poly (A)+ RNA was used to monitor gene expression using CodeLink™ Rat Whole Genome Arrays (Applied Microarray, Tempe, AZ) containing 35,129 gene probes. cRNA synthesis was performed using between 10-100 ng of fecal poly (A)+ RNA. Briefly, reverse transcriptase and a T7-oligo dT primer were used for first strand cDNA and DNA polymerase was used for second strand cDNA generation. After IVT incorporating biotinylated nucleotides, purified and fragmented cRNA was hybridized to a Rat Whole Genome Bioarray in an Innova 4080 shaking incubator (New Brunswick, Edison, NJ) at 300 rpm. After hybridization, the arrays were processed as described previously (84). Images of processed arrays were captured on an Axon GenePix Scanner (Arlington, TX).
**High multiplicity aberrant crypt foci (HM ACF) assay**

To determine whether the FO/P diet was able to suppress formation of early preneoplastic lesions of colon cancer (aberrant crypts) compared with CO/C, we collected colon samples from rats 7 wk after the 2nd AOM injection. Colons were opened and placed flat within folded Whatman #1 paper, followed by fixation in 70% ethanol for 24 h. To identify aberrant crypts, tissue was stained in a 0.5% solution of methylene blue for 45 s. The total number of HM ACF (foci containing four or more aberrant crypts) were counted using a 40× objective (108).

**Colon cancer incidence**

Colons from rats terminated 31 wk after the second AOM injection were used to determine tumor incidence. Tumors were counted and tumor bearing tissues were fixed in 4% PFA for 4 h and embedded in paraffin blocks for histological examination. Tumor sections (4 µm) were stained with hematoxylin and eosin, and tumors were classified as adenomas or adenocarcinomas (101).

**In situ apoptosis**

Apoptosis was measured by TdT-mediated UTP-biotin nick end labeling (TUNEL assay) of fragmented pieces of DNA using 4 µm sections of PFA-fixed, paraffin-embedded tissue (Appendix B-4). Apoptotic cells with condensed chromatin, apoptotic bodies and intense brown staining were counted in 50 crypt columns for each animal. The apoptotic index was calculated as 100 times the mean number of apoptotic cells per crypt column divided by the total number of cells per crypt column (101).
Colonocyte proliferation

Cell proliferation was measured using the proliferating cell nuclear antigen (PCNA) assay. Sections (4 μm) of 70% ethanol-fixed, paraffin-embedded tissue were incubated with PCNA monoclonal antibody (Signet Laboratories, Inc., Dedham, MA). Sections were incubated with biotinylated antimouse IgG (Vector Lab, Burlingame, CA) and then stained with diaminobenzidine tetrahydrochloride (DAB; Sigma) (Appendix B-5) and counterstained with hematoxylin. Twenty-five crypt columns were counted per animal. The number of cells per crypt column and the proportion of proliferating cells per crypt column were determined.

qRT-PCR confirmation of fecal microarray data

Four differentially expressed genes of known function and robust sample size from the microarray platform were selected for validation by qRT-PCR using an ABI 7900HT. These genes were B4galt1 for the 24 h time point, Musdhl and Pdgfa (7 wk) and Id3 for the 28 wk time point. In addition, we selected three other non-differentially highly expressed genes with relevance to colon cancer (Ctsb, Tff3, and Txn1, (109-111)). cDNA was synthesized from 2 ng fecal poly (A)+ RNA and amplified using Ovation PicoSL WTA RNA amplification System (NuGen Technologies, San Carlos, CA). PCR was performed using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). Primer sequences are shown in Table 1 (Integrated DNA Technologies, Coralville, IA). Data are presented as the ratio of the expression level in FO/P-fed rats to that of CO/C-fed rats.
Table 1. Primers for confirmatory qRT-PCR

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>GenBank accession number</th>
<th>Primer sequences</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B4galt1</td>
<td>AI502557</td>
<td>Fwd 5’AGCAGTGACGCCTAATGG Rev 5’ TTGTAGCAGGTCTGACTGTTGACCT</td>
<td>73</td>
</tr>
<tr>
<td>Ctsb</td>
<td>NM_022597</td>
<td>Fwd 5’TCGTATGGATTAGAAAGCTTCTG Rev 5’ AGGGTTACGTGGCACCTCAA</td>
<td>112</td>
</tr>
<tr>
<td>Id3</td>
<td>NM_013058</td>
<td>Fwd 5’ GCTGGTTCTTCTGTTTCTTGGGA Rev 5’ ACATGGTTACAGAAAGGTACACCTTCTTCT</td>
<td>116</td>
</tr>
<tr>
<td>Musdhl</td>
<td>NM_138525</td>
<td>Fwd 5’TGTGGTTCTGGCAAGGACATCG Rev 5’ AGGCGGAGACTCTTCTTCTTGGT</td>
<td>142</td>
</tr>
<tr>
<td>Pdgfa</td>
<td>NM_012801</td>
<td>Fwd 5’GAGGAGGAGACGGATGTGAGGT Rev 5’ CACGCACATGGCAATGAAGCA</td>
<td>119</td>
</tr>
<tr>
<td>Tff3</td>
<td>NM_013042</td>
<td>Fwd 5’AGGGTAAACCTACACCCCTGCT Rev 5’ TCTTCTGGACTCCACCTCTATA</td>
<td>66</td>
</tr>
<tr>
<td>Txn1</td>
<td>NM_053800</td>
<td>Fwd 5’GTGTGGACCTTGGAAATGATC Rev 5’ CTACTTCAAGGAAACCCACATTGG</td>
<td>80</td>
</tr>
</tbody>
</table>

Statistical analyses

Gene expression data for the fecal samples were normalized using the two-stage semi-parametric normalization method of Liu et al (112), which is specifically designed for data generated from partially degraded mRNA. Data were analyzed in SAS using a linear mixed model ANOVA procedure to evaluate the diet effect (FO/P vs. CO/C) at each time point. To correct for multiple testing, a false discovery rate (113) was applied. All genes that were differentially expressed (False Discovery Rate $P$-value, $P < 0.05$) between diets from each time point were used for functional categorization and pathway analysis based on gene ontology (Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatics resources) (114). By importing the list of all differentially expressed genes, this program identified gene ontology (GO) categories
showing enrichment for genes in the list and the probability that the GO categories were being significantly affected by diet and time. We chose to study GO categories within the term “biological process” with a filter of enrichment $P$-value less than 0.05.

Phenotypic data were analyzed using ANOVA to determine the effect of diet (FO/P vs. CO/C) on apoptosis, proliferation, and HM ACF. Colon tumor incidence was analyzed by Chi square analysis and reported as the percentage of rats bearing tumors. Values reported are LSmean ± SEM.

**Results**

The goal of this paper was to monitor the protection provided by the combined FO/P diet in terms of changes in global patterns of intestinal gene expression at the initiation, promotion and tumor stages. Gene expression was monitored using microarray procedures and the resulting data were compared with phenotypic data at each of the three time points to determine if the patterns of expression identified by gene ontology analysis were predictive of changes in disease phenotypes detected in these rats.

**Initiation stage**

At the initiation stage of colon tumorigenesis FO/P resulted in higher levels of apoptosis in the colonic crypt compared to CO/C ($P=0.024$, Table 2). Although there was no significant diet effect on the expression of genes involved in apoptosis 24 h after AOM injection, there was a lower expression of cell adhesion genes ($B4galt1$, $Smoc1$, and $Scarb2$) in FO/P rats compared to CO/C at this time point (Table 3).
Table 2. Effects of FO/P on phenotypes at each time point compared to CO/C in rat

<table>
<thead>
<tr>
<th>Item</th>
<th>CO/C</th>
<th>FO/P</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoptotic index (%)</td>
<td>4.36 ± 0.17 (11)</td>
<td>5.59 ± 0.21* (11)</td>
</tr>
<tr>
<td>7 wk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HM ACF</td>
<td>14.08 ± 1.81 (15)</td>
<td>5.78 ± 1.16* (15)</td>
</tr>
<tr>
<td>Proliferative zone (%)</td>
<td>66.42 ± 0.66 (15)</td>
<td>62.79 ± 0.62* (15)</td>
</tr>
<tr>
<td>Apoptotic index (%)</td>
<td>0.09 ± 0.04 (15)</td>
<td>0.27 ± 0.07* (15)</td>
</tr>
<tr>
<td>Total cell # in a crypt</td>
<td>36.09 ± 0.24 (15)</td>
<td>33.03 ± 0.24* (15)</td>
</tr>
<tr>
<td>31 wk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colon tumor incidence (%)</td>
<td>26.83 (41)</td>
<td>10.26* (39)</td>
</tr>
<tr>
<td>Apoptotic index (%)</td>
<td>0.33 ± 0.20 (19)</td>
<td>0.73 ± 0.31* (20)</td>
</tr>
</tbody>
</table>

1 Data are LSmean ± SEM. The number in parentheses indicates the number of animals.
2 The proliferative zone was calculated as 100 times the position of the highest labeled cell divided by the number of cells per crypt column.
*Different from CO/C, P < 0.05

**ACF stage**

Rats receiving the FO/P diet had fewer HM ACF than did rats receiving the CO/C diet (P=0.0002, Table 2). In addition, the extent of the proliferative zone was lower in rats receiving the FO/P diet compared to the CO/C diet (P=0.0001, Table 2). Relative to observations from rats consuming the CO/C diet, rats consuming the FO/P diet had an elevated apoptotic index (P=0.027, Table 2). The smaller number of cells in the crypt in FO/P group (P=0.0001, Table 2) is likely attributed to both a suppression of cell proliferation and induction of apoptosis. In contrast to the initiation stage, at the HM ACF stage, there were 602 genes that were differentially (false discovery rate P-value <0.05) expressed as a function of diet. Upon completion of GO analyses, 80 biological process categories were found to be significantly enriched (Table 4).
Table 3. Diet-induced differential gene expression at the initiation stage detected in fecal-derived exfoliated cells.

<table>
<thead>
<tr>
<th>GenBank accession</th>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Relative expression</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>AI502557</td>
<td>B4galt1</td>
<td>Udp-gal:beta1,4-galactosyltransferase, polypeptide 1</td>
<td>0.37</td>
<td>cell adhesion</td>
</tr>
<tr>
<td>NM_001002835</td>
<td>Smoc1</td>
<td>Sparc-related modular calcium binding protein 1</td>
<td>0.60</td>
<td>promotes cell-substrate adhesion</td>
</tr>
<tr>
<td>CB726708</td>
<td>Scarb2</td>
<td>Scavenger receptor class B, member 2</td>
<td>0.05</td>
<td>cell adhesion</td>
</tr>
<tr>
<td>BI281095</td>
<td>NULL</td>
<td>UI-DD0-hzrb-09-0.UiI1 UI-R-DD0 cDNA clone</td>
<td>2.96</td>
<td></td>
</tr>
<tr>
<td>BF404993</td>
<td>NULL</td>
<td>UI-CA1-bio-b-08-0.UiI1 UI-R-CA1 cDNA clone</td>
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<td>BF398623</td>
<td>NULL</td>
<td>UI-R-BS2-ber-a-01-0.UiI1 UI-R-BS2 cDNA clone</td>
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<tr>
<td>BF523734</td>
<td>NULL</td>
<td>UI-R-Y0-vb-g-07-0.UiI1 UI-R-Y0 cDNA clone</td>
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<tr>
<td>CB738608</td>
<td>NULL</td>
<td>AMGNNUC:MRPE3-00069-A3-A placenta embryo D17 (10379) cDNA clone</td>
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<td>BF403189</td>
<td>NULL</td>
<td>UI-CA0-bsh-g-02-0.UiI1 UI-R-CA0 cDNA clone</td>
<td>0.12</td>
<td></td>
</tr>
</tbody>
</table>

1 Relative expression represents FO/P divided by CO/C expression level.

Among the 80 clusters, five were directly involved with cell cycle regulation (GO:0007049 cell cycle, GO:0022402 cell cycle process, GO:0000074 regulation of progression through cell cycle, GO:0051726 regulation of cell cycle, and GO:0000079 regulation of cyclin-dependent protein kinase activity). Because four of these categories are a subset of the parent category of cell cycle (GO:0007049), we focused on the parent category in order to include the maximum number of differentially expressed genes.

The FO/P diet yielded almost uniformly lower levels of expression of both cell cycle promoters and suppressors in this cell cycle category (Table 5).
Table 4. GO terms significantly overrepresented in genes altered by the FO/P diet compared to the CO/C diet at the ACF stage.

<table>
<thead>
<tr>
<th>GO number</th>
<th>GO term (biological process)</th>
<th>Enriched genes in list</th>
<th>Total genes in list</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0016043</td>
<td>Cellular component organization and biogenesis</td>
<td>81</td>
<td>2340</td>
<td>2.16E-06</td>
</tr>
<tr>
<td>GO:0047496</td>
<td>Vesicle transport along microtubule</td>
<td>4</td>
<td>6</td>
<td>1.73E-04</td>
</tr>
<tr>
<td>GO:0043283</td>
<td>Biopolymer metabolic process</td>
<td>105</td>
<td>3698</td>
<td>2.56E-04</td>
</tr>
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**Tumor stage**

Similar to the reduction of early preneoplastic lesion numbers, colon tumor incidence evaluated 31 wk after the second AOM injection, was lower in FO/P fed rats than in CO/C rats (Table 2). Part of the protection against tumor formation may be attributable to the enhanced apoptotic index in the FO/P rat colons, compared to those from rats consuming CO/C (Table 2), which was elevated at all three stages of the tumorigenic process.

At the tumor stage, 81 genes were differentially expressed in response to diet, and 13 biological processes were identified by GO analysis as being enriched. Of the 13 categories, six were associated with apoptosis (Table 6). Among the remaining 75
differentially expressed genes, we identified 16 genes known to be involved in signal transduction, and tumor development, progression and invasion (Table 7).

Table 5. Diet-induced differential gene expression at the ACF stage detected in rat fecal-derived exfoliated cells - relative expression of genes in the cell cycle category (GO:0007049)

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<td>Ccnk</td>
<td>Cyclin K</td>
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<td>Ccnl1</td>
<td>Cyclin l1</td>
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<td>Cyclin T2 (predicted)</td>
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1 Relative expression represents FO/P divided by CO/C expression level. All genes have a p-value less than 0.05, indicating that genes were differentially expressed between diets.
Table 6. GO terms significantly overrepresented in genes altered by the FO/P diet compared to the CO/C diet at tumor stage

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Table 7. Diet-induced differential gene expression at the tumor stage detected in rat fecal-derived exfoliated cells

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**Apoptosis from GO category**

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<td>BI302754, NM_013058</td>
<td>Id3</td>
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<td>AA999104</td>
<td>Fem1b</td>
<td>Feminization 1 homolog b (C. elegans) (predicted)</td>
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<td>Homeodomain interacting protein kinase 2 (predicted)</td>
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<td>NM_020302</td>
<td>Adm3</td>
<td>A disintegrin and metalloprotease domain 3 (cytitestin)</td>
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</tbody>
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<sup>1</sup> Relative expression represents FO/P divided by CO/C expression level. All genes have a p-value less than 0.05, indicating that genes were differentially expressed by diet.

<sup>2</sup> Y indicates genes included in the membrane category (GO:0016020).
**qRT-PCR confirmation of fecal microarray**

To validate the fecal microarray data, we performed qRT-PCR on select genes using the same fecal poly (A)+ RNA isolates. The regression between fecal microarray and qRT-PCR results demonstrate a reasonable degree of similarity in the pattern of expression for the seven genes selected for this comparison ($R^2=0.87$, **Figure 3**).

![Figure 3. Correlation of fecal microarray and qRT-PCR results within a subset of genes. Data are expressed as the level of gene expression from feces-derived exfoliated cells of FO/P rats relative to CO/C rats ($R^2 = 0.87$)
Discussion

Most chemoprevention studies are targeted to a single time point in the carcinogenic process. We used a non-invasive technique that permits the isolation of eukaryotic mRNA from exfoliated colonocytes in fecal material (29, 105-107) in order to monitor temporal changes in gene expression. The purpose of the current study was to determine; 1) how diet influences the expression of genes at three discreet stages of tumorigenesis, and 2) if the differences were reflective of the changes in phenotypes measured at those time points.

At the initiation stage of colon tumorigenesis there were only three annotated genes, which were differentially expressed as a function of diet. The low number of diet-induced differential expression at the initiation stage was not unexpected as we have previously shown a relatively small number of differentially expressed genes between diets 12 h after AOM injection (84). The small number of differences between the diets could be explained by the extensive effect of AOM on the colonic epithelium, which prevented detection of diet effects on gene expression. These three genes are all involved in maintaining cell adhesion and it is known that cell adhesion to basement membranes prevents cell death (103), suggesting that the FO/P diet would facilitate apoptosis induction. The lower relative expression of cell adhesion related genes in cells after AOM injection likely contributes to the ability of FO/P rats to effectively eliminate cells with DNA damage (83, 115). Whether the elimination is through the induction of apoptosis or by cell sloughing (103, 116), these changes in gene expression would
explain some of the chemoprotection provided by a FO/P diet, relative to the effects observed with a CO/C diet.

At the HMACF stage, we found that 602 genes were differentially expressed as a function of diet. This suggests that as carcinogenesis progresses to this stage, the ability of diet to affect gene expression and thereby provide a chemoprotective effect is enhanced as reflected by the number of phenotypic changes that are detected (i.e. apoptosis, proliferative zone, and HM ACF). Gene ontology analysis of the 602 differentially expressed genes revealed that cell cycle regulation was affected by a FO/P diet compared to CO/C. Changes in the expression of genes involved in cell cycle regulation are critical to the promotion of colon carcinogenesis (117). The cyclin-CDK complex initiates the phosphorylation of $Rb$, stimulating the regulation of cell cycle progression (102). Relative to the expression in the CO/C rats, expression of cyclin $G1$, $K$, $L1$, $T2\_predicted$ as well as $cdc34\_predicted$ were lower in rats consuming the FO/P diet. $Rbl2$ is known to control progression from G0 into the G1 phase of the cell cycle, and the level of its expression was lower in rats consuming a diet containing FO/P compared to CO/C. However, the phosphorylation of $Rbl2$ is needed to release E2F and activate the cell cycle. FO/P resulted in lower levels of expression of $CDK$ and $GSK3b$, which is a reported kinase of $Rbl2$ (118) and is expected to reduce the degree of phosphorylation of $Rbl2$. Another promoter of cell cycle activity is $Ruvbl1$, which is up-regulated in human colon cancers. $Ruvbl1$ enhances the transcription of Wnt target genes by interacting with $\beta$-catenin (119). In this experiment, the FO/P diet down-regulated the expression of $Ruvbl1$ compared to the CO/C diet, which suggests the FO/P diet may
inhibit Wnt signaling. These findings suggest that FO/P diet could suppress the uncontrolled cell proliferation that occurs in colon cancer cells at the ACF stage in part by modulating the expression of genes that are essential for cell cycle progression.

In addition to the suppression of cell proliferation, we previously reported on the cell-specific expression of O\(^6\) -methylguanine DNA methyltransferase (MGMT, DNA repair enzyme). The expression of MGMT in fish oil-fed rats was 4-fold higher than corn oil-fed rats in areas of colon crypts where apoptosis typically occurs (83). Pms2 is known to be involved in DNA mismatch repair systems, and mutation of Pms2 is documented to cause hereditary nonpolyposis colorectal cancer (120). In the current study, we found that the expression of Pms2\(_{predicted}\) was 3-fold higher in FO/P rats compared to CO/C rats. We also observed that FO/P significantly enhanced apoptosis compared to CO/C (Table 2). Therefore, the FO/P diet may facilitate removal of DNA damaged cells by increasing DNA repair and apoptosis.

At the tumor stage, the modulation of expression of several genes involved in apoptosis occurred in concert with the induction of apoptosis in the FO/P rats. Of the six differentially expressed genes involved in the apoptosis pathway, Mmp2 expression was increased in the colon of CO/C tumor-bearing rats. This is noteworthy because Mmp2 has been implicated in colon cancer invasion (121). Expression of Id3, an apoptosis inducer (122), was higher in FO/P rats compared to CO/C, whereas Tmem23 and Hgf, both apoptosis inhibitors (123, 124), were lower in FO/P rats compared to CO/C. This pattern of gene expression is consistent with the apoptotic phenotype observed across all
time points, indicating at a molecular level why the FO/P diet is more effective in promoting apoptosis than the CO/C diet.

The expression of 11 genes (e.g., Slc8a1, Dupd1, Ppp1r7, Mfn1, Stx1a, Smoc1, Snip, Nrn1, Il23a, Il6ra, and Pthr2) involved in several signal transduction pathways was down-regulated in FO/P fed rats compared to CO/C, suggesting that FO/P is capable of attenuating multiple signaling pathways at the tumor stage. For example, Mfn1, which is a transmembrane GTPase, is one of the genes down-regulated by FO/P compared to CO/C. Mfn1 mediates mitochondrial fusion, and elevated expression of Mfn1 has been demonstrated to increase the resistance of cells to death stimuli (125). With regard to this mechanism of action, 11 genes in the membrane category (GO:0016020) were differentially expressed at the tumor stage (indicated by “Y” in Table 2). This may be explained by the incorporation of DHA, a bioactive component of fish oil, into both plasma and mitochondrial membranes (80). Indeed, we and others have demonstrated that (n-3) PUFA promote an oxidation-reduction imbalance in the intestine (126-129). Recently, we have demonstrated that DHA promotes mitochondrial oxidative stress and increases mitochondrial Ca^{2+} levels, which directly induce apoptosis in colonocytes (96, 97).

Tumor related genes involved in tumor formation, progression, and invasion were favorably modulated by FO/P consumption compared to CO/C. For example, the expression of Cyp2s1 was down-regulated by FO/P as compared to CO/C. This gene encodes for one of the cytochrome P450 superfamily members and plays a pivotal role in the oxidative metabolism of xenobiotics such as carcinogens. A study designed to
identify markers of colon cancer prognosis demonstrated that the expression of \textit{Cyp2s1} was significantly higher in primary colon cancers than in normal colon tissue (130). \textit{Adam3}, an indicator of tumor invasion, was also down-regulated in FO/P rats compared to CO/C (131, 132). In contrast, \textit{Brms1}, a tumor suppressor gene, was up-regulated by FO/P compared to CO/C. Similarly, FO/P rats had twice the level of \textit{Rbbp6} (also designated \textit{P2P-R}) expression as CO/C rats, and overexpression of this gene results in mitotic arrest at prometaphase and mitotic apoptosis (133).

Existing studies with the AOM model of colon carcinogenesis have reported gene expression at discreet points in the tumorigenic process (46, 11). Those studies also found changes in expression of genes involved in cell adhesion (46), apoptosis or cell cycle (11). The unique contribution of the current study is that we found that diet differentially affects apoptotic genes, depending upon the stage of tumorigenesis. Therefore, we have discovered time-specific effects of diet that contribute to chemoprotection against colon cancer. Interestingly, although the FO/P diet promoted apoptosis at all three time points, it differentially impacted gene expression at each stage. These data are consistent with recent observations (47), indicating a clear time-dependent, divergent regulation of gene expression signatures in response to the fatty acid content of the diet.

We have demonstrated the feasibility of monitoring gene expression over time using an mRNA-based noninvasive technique. This makes it possible to determine the mechanisms whereby a chemopreventive diet may inhibit colon carcinogenesis as well as to monitor human disease progression and identify critical time points for potential
diet intervention. In this study, we have identified differentially expressed genes involved in apoptosis and/or cell proliferation at three time points during colon carcinogenesis. At the initiation stage, there were few differential effects of diet on gene expression. At the promotion stage, the expression of many more genes was affected by diet, suggesting this stage is more susceptible to the FO/P dietary intervention. Gene ontology pathways enriched at this stage include cell proliferation. However, at the tumor stage, the main gene expression pathway affected was principally associated with apoptosis. Consequently, the central mechanism by which FO/P produces a chemoprotective effect is through changes in gene expression that enhance cell cycle regulation and apoptosis throughout tumorigenesis.
CHAPTER III

A CHEMOPROTECTIVE FISH OIL/PECTIN DIET ENHANCES APOPTOSIS VIA BCL-2 PROMOTER METHYLATION IN RAT AZOXYMETHANE-INDUCED CARCINOMAS

We have demonstrated that diets containing FO/P reduce colon tumor incidence relative to those containing CO/C in part by inducing apoptosis of DNA-damaged colon cells. Relative to FO/P, CO/C promotes colonocyte expression of the anti-apoptotic modulator, Bcl-2, and Bcl-2 promoter methylation is altered in colon cancer. To determine if FO/P, compared with CO/C, limits Bcl-2 expression by enhancing promoter methylation in colon tumors, we examined Bcl-2 promoter methylation, mRNA levels, colonocyte apoptosis, and colon tumor incidence in azoxymethane (AOM)-injected rats. Rats were provided diets containing FO/P or CO/C, and were terminated 16 and 34 wk after AOM injection. DNA isolated from PFA-fixed colon tumors and uninvolved tissue was bisulfite modified and amplified by qRT-PCR to assess DNA methylation in Bcl-2 CpG islands. FO/P increased Bcl-2 promoter methylation (p=0.009) in tumor tissues and colonocyte apoptosis (p=0.020) relative to CO/C. An inverse correlation between Bcl-2 DNA methylation and Bcl-2 mRNA levels was observed in the tumors. We conclude that dietary FO/P promotes apoptosis in part by enhancing Bcl-2 promoter methylation. These Bcl-2 promoter methylation responses, measured in vivo, contribute to our understanding of the mechanisms involved in chemoprevention of colon cancer by diets containing FO/P.
Introduction

Epigenetic regulation of gene transcription occurs through mechanisms that include DNA methylation and histone modification (34, 35). DNA methylation occurs primarily at cytosine-guanosine dinucleotides (CpG) by the addition of a methyl group on the 5’ carbon position of cytosine. CpG islands are CpG-rich regions in or near the promoter region of genes, that when unmethylated allow the transcription of genes (35). Aberrant methylation of promoter CpG islands has been accepted as a crucial contributor to cancer development (134, 135) because the extent of promoter methylation in tumors is associated with disease progression, recurrence, and survival rates in colon cancer (136, 137). A hallmark of tumorigenesis is an inhibition of apoptosis, and genes involved in apoptosis can be transcriptionally regulated through DNA methylation (43). Importantly, aberrant DNA methylation is potentially reversible by natural agents in the diet, such as soy isoflavones, apple polyphenols, green tea polyphenols, and biologically active compounds present in vegetables (68, 70, 72, 138), which may lead to induction of apoptosis in the colon.

We have previously demonstrated that the combination of FO/P, compared with a control diet containing CO/C, promoted apoptosis during colon cancer development (85). Fish oil is high in n-3 polyunsaturated fatty acids (PUFA), i.e., eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), whereas corn oil is high in the n-6 fatty acid, linoleic acid (LA, 18:2n-6). Pectin is a highly fermentable fiber that yields butyrate upon microbial fermentation, whereas cellulose is poorly fermented. One
of the mechanisms by which FO/P enhances apoptosis is suppression of \textit{Bcl-2} levels, an anti-apoptotic mediator (86, 139). \textit{Bcl-2} obstructs the intrinsic apoptotic pathway by preventing the release of cytochrome C from the mitochondria, thereby suppressing downstream caspase activation (140). In leukemia cells, \textit{Bcl-2} is hypomethylated leading to high \textit{Bcl-2} expression (46). These observations are relevant because high levels of \textit{Bcl-2} are observed in the metastatic state of various types of cancer including colon cancer (13). Therefore, we hypothesized that the suppression of \textit{Bcl-2} expression in response to a FO/P diet may result from a previously unidentified modulation of the \textit{Bcl-2} promoter region. To test this, we determined the methylation status of specific CpG dinucleotides of the \textit{Bcl-2} promoter region and the transcription level of \textit{Bcl-2}. This study provides evidence that FO/P modulates apoptosis in part by epigenetic regulation of \textit{Bcl-2} expression in rat AOM-induced colon carcinomas.

**Materials and methods**

**Animals and study design**

The animal use protocol was approved by the Institutional Animal Care and Use Committee of Texas A&M University and conformed to National Institutes of Health (NIH) guidelines. Ninety-six male Sprague-Dawley rats (Harlan Teklad, Madison, WI) were housed individually in a temperature and humidity controlled animal facility with a 12 h light/dark cycle. After 1 wk of acclimation and 1 wk receiving the experimental diets, rats were injected with azoxymethane (AOM; Sigma, St Louis, MO, 15 mg/kg
body weight) twice, 1 wk apart. Twenty-two rats (CO/C n = 12, FO/P n = 10) were terminated at 16 wk and 74 rats (CO/C, n = 41; FO/P, n = 33) were used for final tumor analysis at 34 wk after the second AOM injection. Rats were killed by CO$_2$ overdose and cervical dislocation. Samples from 16 wk were used for apoptosis and DNA methylation analyses and those from 34 wk were used for apoptosis, DNA methylation and gene expression analyses (Figure 4).

**Diets**

Rats were provided a diet containing either FO/P or CO/C as previously described (101). The FO/P diet contained higher amounts of n-3 PUFA (EPA and DHA).
and pectin, a highly fermentable fiber. The CO/C diet had higher amounts of n-6 PUFA (LA) and cellulose, a poorly fermented fiber. The fish oil diet included 3.5 g corn oil/100 g diet to meet essential fatty acid requirements. All diets contained oils at 15% by weight and 30% of calories, an amount that corresponds to current human dietary recommendations. The amount of fiber in the diet was 6% by weight, which is equivalent to 30 g/d for humans, also within the recommended range for humans. The antioxidant levels in the FO/P and CO/C diets were 1.5 g/kg α-tocopherol, 1 g/kg γ-tocopherol, and 0.025 % tertiary butylhydroquinone. Fresh diets were provided daily to prevent lipid oxidation.

Colon cancer incidence

Colons from rats terminated 34 wk after the second AOM injection were used to determine tumor incidence. Tumors were counted and tumor tissues were fixed in 4% PFA for 4 h and embedded in paraffin blocks for histological examination. Tumor sections (4 µm) were stained with hematoxylin and eosin, and tumors were classified as adenomas or adenocarcinomas (101).

In situ apoptosis

Apoptosis was measured by TdT-mediated UTP-biotin nick end labeling (TUNEL assay) of fragmented pieces of DNA using 4 µm sections of PFA-fixed, paraffin-embedded tissue. Apoptotic cells with condensed chromatin, apoptotic bodies and intense brown staining were counted in 25 crypt columns for each animal. The apoptotic index was calculated as 100 times the mean number of apoptotic cells per crypt column divided by the mean number of cells per crypt column (101).
**DNA extraction and preparation**

Genomic DNA was extracted from 10 μm PFA-fixed colon carcinomas and non-involved tissue sections using a QIAamp DNA FFPE tissue kit (Qiagen, Valencia, CA). In addition, to prepare a universally methylated reference sample, rat liver genomic DNA was treated with CpG Methyltransferase (M.SssI, New England Biolabs, Ipswich, MA), which methylates cytosines in CpG dinucleotides with S-adenosyl methionine (SAM) as a methyl donor. Briefly, genomic DNA of rat liver was treated with M.SssI (0.05 units/μl final concentration) and SAM overnight, followed by another overnight incubation with an additional M.SssI (0.09 units/μl final concentration) and SAM (141). The M.SssI-treated DNA was purified using a DNA Clean and Concentrator kit (Zymo Research, Irvine, CA) (Appendix B-7).

**Sodium bisulfite modification and DNA recovery**

Two μg of genomic DNA or M.SssI treated DNA was sodium bisulfite modified using a Zymo EZ DNA Methylation Kit (Zymo Research, Irvine, CA) as per the manufacturer’s instructions. Sodium bisulfite converts unmethylated cytosines to uracils, but does not affect the methylated cytosines (Figure 5).

**qPCR detection of Bcl-2 promoter methylation status**

After sodium bisulfite conversion, genomic and M.SssI treated DNA were amplified by fluorescence-based qPCR using an ABI 7900 unit. Serial dilutions of M.SssI treated DNA were included on each plate to generate a standard curve. PCR amplification was performed with a final reaction mixture of 25 μl consisting of 600 nM of each primer, 200 nM probe, 200 μM dNTPs, 3.5 mM MgCl₂, 10× TaqMan buffer
Figure 5. DNA sequence conversion by bisulfite modification without Uracil DNA glycosylase (AMPerase), 10× stabilizer, and water. Bisulfite-converted DNA was amplified at 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min (Appendix B-8) (142). Primers and probes, designed specifically for bisulfite-converted DNA, were used for Bcl-2 and Col2a1, a reference gene to normalize for input DNA. Bcl-2 primers and probe were designed to contain 7 CpG dinucleotides in a CpG island located in the first exon of Bcl-2 (Figure 6). Primers and the probe were designed in a region of the Col2a1 gene that lacks any CpG dinucleotides to allow for equal amplification, regardless of methylation levels. The percentage of fully methylated molecules at a specific locus was calculated by dividing the Bcl-2:Col2a1 ratio of a sample by the Bcl-2:Col2a1 ratio of M.SssI-treated DNA and multiplying by 100. The primer and probe sequences are listed in Table 8. (Appendix B-6)
Figure 6. Genomic structure of rat Bcl-2 and the location of primers and probe for DNA methylation. The | and ■ represent CpG dinucleotides.

Table 8. Primer and probe sequence of promoter methylation

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<th>Gene</th>
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<th>Reverse primer 5' to 3'</th>
<th>Probe sequence 5' to 3'</th>
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<td>Bcl-2</td>
<td>NM_016993</td>
<td>496-573</td>
<td>GGATATTTTTGT AAAGTCGCGAC</td>
<td>ACCTATAATCC ACCTAACCCCTC CG</td>
<td>6FAM-CTAAAAATAAC TTCTCTCGTCGCT-MGB</td>
</tr>
</tbody>
</table>
| Col2a1 | NM_012929 | 13,169-13,244 | GGTATTTTTGT GTTTTGAAGAG TAGT TG | CTTTCTCTCT CCTTAAAACTCC AA | MGB refers to a Minor Groove Binder non-fluorescent quencher in the 3' terminus of the probe (MGBNFQ).

RNA extraction and transcription level measurement using qRT-PCR

Total RNA was extracted from colon carcinomas using Totally RNA (Ambion, Grand Island, NY), followed by treatment with DNase. cDNA was synthesized from 2 µg of total RNA using oligo dT primer and SuperScript II reverse transcriptase (Invitrogen, Grand Island, NY) (Appendix B-3). qRT-PCR was performed using an ABI 7900 and TaqMan gene expression assays (Applied Biosystems, Foster City, CA) to
quantify mRNA expression levels of *Bcl-2*. The expression level of *Bcl-2* (Rn99999125_m1) was normalized to Eukaryotic 18S rRNA expression (Hs99999901_s1) (139). Accuracy of the assay was validated by establishing linearity of response for each assay. Negative controls consisted of reverse transcription reaction components without RT enzyme.

**Statistical analyses**

Data were analyzed using independent samples t-test to determine the effect of diet (FO/P vs. CO/C) on apoptosis and *Bcl-2* methylation. Colon tumor incidence was analyzed by Chi square analysis and reported as the percentage of rats bearing tumors. The relationship between DNA methylation and gene expression was determined by regression analysis. The values are reported as means ± SEM. Differences between treatments were regarded as significant when P values were <0.05.

**Results**

**FO/P diet promotes apoptosis and reduces colon tumor incidence**

Rats receiving the FO/P diet had a significantly lower colon tumor incidence compared with those receiving the control CO/C diet (p=0.016, **Figure 7A**). In addition, rats consuming the FO/P diet had a higher (p=0.028) number of apoptotic cells per crypt column (0.85 ± 0.17) compared to those consuming the CO/C diet (0.47±0.08). This resulted in an elevated apoptotic index (percentage of apoptotic cells in crypt column) in
the non-tumor (uninvolved) regions of the colon in FO/P rats (means of data at 16 and 34 wk after AOM injection), compared to CO/C (p=0.020, Figure 7B).

Figure 7. Colon tumor incidence (n = 74) (A), Average of apoptotic index values measured at 16 wk and 34 wk after AOM injections (n = 40) (B). The data are means ± SEM. *Different from CO/C, P<0.05. FO/P = fish oil/pectin diet and CO/C = corn oil/cellulose diet. Data for this figure is found in Appendix Table A-1.

**FO/P enhances Bcl-2 promoter methylation in tumors at 34 wk after AOM injections**

At the 16 wk time point, Bcl-2 promoter methylation tended to decline with the FO/P diet, but this effect was not significant (CO/C 0.94 ± 0.16, FO/P 0.65 ± 0.12, p=0.083, Figure 8A). In contrast, at the 34 wk time point, FO/P enhanced Bcl-2 promoter methylation, which was significant (p=0.009) in carcinomas (CO/C 0.57 ± 0.06, FO/P 0.91 ± 0.19) but not in uninvolved colonic mucosa (CO/C 0.37 ± 0.04, FO/P 0.50 ± 0.11) (Figure 8B). Bcl-2 promoter methylation was lower in uninvolved tissues at 34
wk (0.42 ± 0.05) compared with those of 16 wk (0.79 ± 0.10) (p=0.001, Figure 8C),
which likely reflects the continued progression of tumorigenesis.

Figure 8. Relative Bcl-2 promoter methylation 16 wk after AOM injections (6 rats/group) (A), relative Bcl-2 promoter methylation 34 wk after AOM injections (14-25 rats/group) (B), relative Bcl-2 promoter methylation of uninvolved tissues 16 wk and 34 wk after AOM injections (n=12 at 16 wk, n=36 at 34 wk) (C), and relative Bcl-2 gene expression in carcinomas (3-4 rats/group) (D). The data are means ± SEM. *Different from CO/C, P<0.05. FO/P = fish oil pectin diet and CO/C = corn oil/cellulose diet. Data for this figure is found in Appendix Table A-1 and A-2.
**Bcl-2 gene expression tends to be associated with reduced promoter methylation**

Our hypothesis was that FO/P would increase *Bcl-2* promoter methylation, leading to lower gene transcription. In carcinomas collected at 34 weeks, *Bcl-2* promoter methylation was elevated in rats receiving the FO/P diet, which occurred in concert with numerically lower *Bcl-2* transcription in carcinomas compared with CO/C (p=0.13, **Figure 8D**). When the extent of *Bcl-2* promoter methylation was compared with *Bcl-2* expression in carcinomas of rats consuming FO/P or CO/C, the anticipated negative correlation (slope= -0.005, $R^2=0.16$) was observed even though the limited number of observations precluded obtaining a significant relationship (p=0.25, **Figure 9**).

![Figure 9. Correlation of Bcl-2 promoter methylation and gene expression in carcinomas (slope= -0.005, $R^2=0.16$, p=0.25) (Image)](image-url)
Discussion

DNA methylation is a well-established epigenetic mechanism that leads to changes in gene expression without DNA sequence alterations. Aberrant hypermethylation of promoters is a recognized mechanism by which the expression of tumor suppressor genes is inhibited during colon cancer development (134, 135). In contrast to hypermethylation of tumor suppressor genes, a region in the first exon of Bcl-2 oncogene is completely demethylated allowing high-levels of Bcl-2 expression in B-cell chronic lymphocytic leukemia (46). Aberrant promoter methylation has been proposed as a prognostic marker of cancers, and the extent of methylation in a tumor is associated with disease progression, recurrence, and survival rates in various types of cancer (143-145). For instance, colon cancer patients with promoter hypermethylation exhibit shorter cancer-specific survival compared with those without promoter hypermethylation (137).

The combination of FO/P has been extensively studied to identify its chemoprotective mechanisms in colon carcinogenesis, including its ability to synergistically induce apoptosis (96, 97, 146). Apoptosis can be induced by changes in the regulation of histone acetylation, another epigenetic means of regulating gene transcription (85, 92). In addition, we have recently demonstrated that FO/P modulates non-coding microRNA in AOM-induced colon cancer, another epigenetic mechanism whereby FO/P regulates gene transcription (99, 147).
In this study, we investigated the ability of FO/P to modulate methylation-based epigenetic regulation of the \textit{Bcl-2} gene in the AOM-induced colon cancer model. Methylation of the \textit{Bcl-2} promoter was targeted because we previously demonstrated that the combination of FO/P, compared with CO/C, suppressed the level of \textit{Bcl-2} expression, which occurred in parallel with the induction of colonocyte apoptosis (139). Therefore, the goal of this paper was to determine if a diet enriched in FO/P modulates the level of \textit{Bcl-2} gene expression by enhancing promoter methylation \textit{in vivo}.

As we previously reported (95), the incidence of colon tumors in rats consuming a FO/P diet was lower than in those consuming a CO/C diet. Part of the protection against tumor formation may be attributable to the enhanced apoptotic index in the rats consuming FO/P compared with those from rats consuming CO/C (\textbf{Figure 7}).

Our results indicated that AOM-induced colon carcinomas exhibited a higher relative methylation of the \textit{Bcl-2} promoter in rats consuming a chemoprotective FO/P diet, compared to CO/C. Interestingly, this effect was not found in the uninvolved tissue sections. Although no other studies have investigated the effect of diet on methylation of \textit{Bcl-2}, several reports have demonstrated dietary effects on DNA methylation in other genes within cancer cells or animal models of cancer. For example, Nandakumar et al. demonstrated that green tea polyphenols restored the expression of the tumor suppressor genes \textit{Cip1/p21} and \textit{p16^{INK4a}} in human skin cancer cells by suppressing DNA methylation (68). Fang et al. reported that genistein, a soy isoflavone, reversed hypermethylation and reactivated \textit{RARβ}, \textit{p16^{INK4a}}, and \textit{MGMT} in human esophageal squamous cancer cells (70). Using the AOM-treated Apc\textsuperscript{Min/+} mouse model, Volate et al.
determined that consumption of low concentrations of green tea led to demethylation of the \textit{RXR\textalpha} promoter region, which restored \textit{RXR\textalpha} expression in colon mucosa (69). The current study is the first to demonstrate \textit{Bcl-2} promoter methylation regulation by diet \textit{in vivo}.

Babidge et al. demonstrated that specific CpG dinucleotides in the \textit{Bcl-2} gene were methylated in 20\% of human colorectal tumors, but that four CpG dinucleotides in the promoter region were not methylated in any colorectal tumors (47). In the current study, \textit{Bcl-2} promoter methylation declined from the levels observed at the intermediate stage of tumorigenesis (16 wk) to those found in the non-involved tissues at 34 wk after AOM injections. By modulating the transcriptional regulation of \textit{Bcl-2} at the tumor stage, levels of apoptosis were suppressed as is recognized to occur in colon cancer (86, 139, 148). However, the FO/P diet was able to enhance \textit{Bcl-2} promoter methylation in carcinomas, which should enhance apoptosis. Indeed, Koelink et al. demonstrated that decreased tumor apoptosis was associated with a significantly higher probability of disease recurrence in colon tumors (149). Since cancer cells are resistant to the induction of apoptosis, reversing \textit{Bcl-2} promoter hypomethylation by a FO/P diet could offer a therapeutic approach.

The levels of \textit{Bcl-2} transcripts were numerically lower in tumors from rats consuming a diet containing FO/P compared with CO/C. Even though the difference between diet groups was not significant, the pattern of response was similar to a previous report (139), and reflects the elevated methylation state in carcinomas from the FO/P rats in this study. An extensive comparison could not be made due to the relatively small
number of samples for which both DNA and RNA was available. However, other researchers have demonstrated that diets rich in fish oil prevented the expression of Bcl-2 in the colon cancer cells (148, 150), as well as other cell types (151). Moreover, this result is comparable to those of Zhu et al., who reported that the Bcl-2 gene promoter is hypomethylated in colorectal cancer tissues, and that Bcl-2 methylation was inversely correlated with Bcl-2 protein expression in those tissues (152). Similar results were also demonstrated by Carvalho et al. who found that Bcl-2 mRNA expression was inversely correlated with promoter methylation in advanced stage prostate carcinomas (153).

In summary, these data suggest that dietary FO/P promotes apoptosis in part by enhancing Bcl-2 promoter methylation in colon tumors relative to a CO/C diet. This finding contributes to our understanding of the mechanisms whereby diets containing FO/P are chemoprotective against colon cancer. In order to fully appreciate how a diet containing FO/P modulates DNA methylation during colon carcinogenesis, the effects of FO/P on DNA methyltransferases (the enzymes responsible for DNA methylation) should be investigated.
CHAPTER IV
NUTRIEPIGENETIC REGULATION OF APOPTOSIS-RELATED GENES
BY DHA AND BUTYRATE

Aberrant epigenetic changes in chromatin, such as DNA hypermethylation and histone deacetylation, contribute to the transcriptional regulation of genes involved in apoptosis. We have demonstrated that decosahexaenoic acid (DHA, 22:6 n-3) and butyrate enhance colonocyte apoptosis. To determine if DHA and/or butyrate elevate apoptosis through epigenetic mechanisms thereby restoring the transcription of apoptosis-related genes, we examined global methylation, gene specific promoter methylation of 24 apoptosis-related genes, apoptosis, transcription levels of Cideb, Dapk1, and Tnfrsf25 and global histone acetylation in HCT-116 cell line. Cells were treated with combinations of (50 µM) DHA or linoleic acid (LA, 18:2 n-6), (5 mM) butyrate or an inhibitor of DNA methyltransferases, 5-aza-2’-deoxycytidin (5-Aza-dC, 2 µM). Among highly methylated genes, the combination of DHA and butyrate significantly reduced the methylation of the proapoptotic Bcl2l11, Cideb, Dapk1, Ltbr, and Tnfrsf25 genes, and increased apoptosis by 180% compared to untreated control. DHA treatment reduced the methylation of Cideb, Dapk1, and Tnfrsf25. These data suggest that the induction of apoptosis by DHA and butyrate are regulated, in part, through changes in the methylation state of apoptosis-related genes.
Introduction

During carcinogenesis, gene transcription is aberrantly regulated via epigenetic changes. These changes include DNA methylation and histone modification and occur primarily in the promoter region of select genes (33, 34). Clinical studies have demonstrated a correlation between DNA methylation and the pathological and epidemiological features of colon cancer (134, 135, 154). Recent data indicate that promoter methylation of proapoptotic genes suppresses transcription, resulting in the cancer cell survival (43). For instance, Dapk1 is a positive mediator of apoptosis, a tumor suppressor candidate, and is known to be heavily methylated in colon and bladder cancer (44, 45). Restoring transcription of tumor suppressor genes by the reversal of these epigenetic processes is considered a latent target of cancer prevention and treatment.

We have previously shown that a combination of fish oil (high in DHA, an n-3 polyunsaturated fatty acid (PUFA)) and the fiber pectin (fermented to short chain fatty acids including butyrate by the colonic microflora) is protective against colon carcinogenesis in part by up-regulating the apoptotic removal of DNA damaged cells (101). In contrast, diets highly enriched in corn oil (high in LA, an n-6 PUFA) suppress apoptosis and promote colon cancer (84). We have demonstrated that apoptosis induction by the combination of fish oil and pectin is in part contingent upon DHA incorporation into mitochondrial phospholipids (78), while butyrate functions as an energy substrate and histone deacetylase inhibitor (56). Another potential mechanism
involved in tumor suppression by fish oil/pectin is through the transcriptional regulation of key tumor suppressors or oncogenes. In a previous study, we demonstrated the effect of fish oil/pectin on global changes in gene expression profiles in carcinogen injected Sprague-Dawley rats. One result from the pathway analyses of those data was the observation that the expression of genes that promote apoptosis was up-regulated in rats consuming the fish oil/pectin diet at the tumor endpoint (85). Moreover, we have reported that the promoter region of Bcl-2, an antiapoptotic mediator, in carcinogen-induced colon tumors is highly methylated which was associated with induction of apoptosis in colonocytes from rats consuming a fish oil/pectin diet (155).

However, it has not been determined if DHA and/or butyrate can directly affect the aberrant promoter methylation of cancer-promoting genes in cancer cells. Therefore, the aim of this study was to investigate the epigenetic regulation of apoptosis-related genes in colon cancer cells exposed to DHA and/or butyrate. For this purpose, we examined global DNA methylation and histone acetylation in combination with gene specific promoter methylation of 24 apoptosis-related genes in HCT-116 human colon cancer cells.

**Materials and Methods**

**Cell culture**

HCT-116 cells were cultured in McCoy’s 5A (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 2 mM GlutaMAX
(Gibco) at 37°C in 5% CO₂. Cells (passages 11-13) were seeded onto 100 mm cell culture dishes or six-well plates at a density of 3.0×10⁴ cells and allowed to attach for 24 h (Figure 10). (Appendix B-9)

Experiment I: Fifty μM bovine serum albumin (BSA)-complexed DHA or LA (Nu Chek Prep. Inc) was used to treat cells for 72 h (94). Cells were co-incubated with 5 mM sodium butyrate (Sigma, St. Louis, MO) during the final 12 h of the fatty acid treatment period (96). 5-Aza-dC (2 μM), a potent inhibitor of DNA methylation (Santa Cruz Biotechnology, Santa Cruz, CA) served as a positive control, and cells were treated for 48 h (Appendix B-11). Negative controls were cells incubated in media without BSA-complexed fatty acids or butyrate (Appendix B-10).

Experiment II: The goal of this experiment was to determine the time-course for gene expression relative to treatment with 5-Aza-dC, fatty acids and/or butyrate. For this purpose, all cells received the assigned treatments at time 0, and gene expression was monitored in cells harvested at 48, 72 and 96 h post treatment initiation. The treatments included 50 μM BSA-DHA or BSA-LA, 5 mM butyrate, or 2 μM 5-Aza-dC.

**Global DNA methylation assay**

Genomic DNA was isolated from HCT-116 cells using QIAamp DNA mini kit (Qiagen, Valencia CA, Appendix B-12). Global methylation was quantified using a MethylFlash methylated DNA quantification kit (Epigentek, Farmingdale, NY). Briefly, 100 ng of genomic DNA was pipetted into multiwell plates coated with a 5-methylcytosine antibody and incubated at 37°C for 90 min. DNA was subsequently
washed and a capture antibody added prior to incubation at room temperature for 30 min. At the end of the incubation period, the capture antibody solution was removed and replaced with an enhancer solution followed by another 30 min incubation at room temperature. The extent of global methylation was measured by reading absorbance at 450 nm. The relative methylation status of each sample was determined using positive and negative controls provided in the kit (Appendix B-13).
**Apoptosis assay**

Apoptosis was measured using Cell Death Detection ELISA plus kit (Roche, Indianapolis, IN). For this assay, the floating cells were collected, washed, lysed and centrifuged to remove intact DNA. The supernatant, which includes both mono- and oligonucleosomes from the cell lysates, was transferred to a streptavidin-coated microplate. Anti-histone antibody and anti-DNA antibodies were added to the wells, in order to promote the binding of the antibody-nucleosome complex to the microplate. After a washing step to remove non-bound cellular debris, the samples were incubated with a peroxidase substrate and the absorbance was measured at 405 nm. Data were normalized with the number of cells to calculate the apoptotic index as previously described (94) (Appendix B-14).

**Gene specific DNA methylation**

DNA methylation of apoptosis-related genes was determined using a Methyl-Profiler DNA methylation PCR array (SABiosciences, Valencia, CA, MeAH-121C-2). Genes included on the array are listed in Table 9. Briefly, 1 µM of genomic DNA from HCT-116 cells was separated into four equal aliquots and treated with: 1) a mock enzyme, 2) a DNA methylation sensitive enzyme, 3) a DNA methylation dependent enzyme, or 4) both the DNA methylation sensitive and dependent enzymes at 37°C overnight. The relative DNA methylation status was determined using Ct values from qRT-PCR as previously described (156). Data are presented as a percentage of cellular DNA containing methylated gene promoters, which included a combination of both the intermediate level of methylation and hypermethylated DNA (Appendix B-15).
Table 9. Gene specific DNA methylation targets using PCR array

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<th>RefSeq accession number</th>
<th>Symbol</th>
<th>Description</th>
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<tbody>
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<td>NM_009684</td>
<td>Apaf1</td>
<td>Apoptotic peptidase activating factor 1</td>
</tr>
<tr>
<td>NM_007522</td>
<td>Bad</td>
<td>BCL-2-associated agonist of cell death</td>
</tr>
<tr>
<td>NM_009736</td>
<td>Bag1</td>
<td>Bcl-2-associated athanogene 1</td>
</tr>
<tr>
<td>NM_007527</td>
<td>Bax</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>NM_009741</td>
<td>Bcl2</td>
<td>B-cell leukemia/lymphoma 2</td>
</tr>
<tr>
<td>NM_007546</td>
<td>Bcl2/11</td>
<td>BCL-2-like 11 (apoptosis facilitator)</td>
</tr>
<tr>
<td>NM_153787</td>
<td>Bclaf1</td>
<td>BCL-2-associated transcription factor 1</td>
</tr>
<tr>
<td>NM_007544</td>
<td>Bid</td>
<td>BH3 interacting domain death agonist</td>
</tr>
<tr>
<td>NM_007546</td>
<td>Bik</td>
<td>Bcl-2-interacting killer</td>
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<td>NM_007465</td>
<td>Birc2</td>
<td>Baculoviral IAP repeat-containing 2</td>
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<tr>
<td>NM_009761</td>
<td>Bnip3l</td>
<td>BCL-2/adenovirus E1B interacting protein 3-like</td>
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<td>NM_009810</td>
<td>Casp3</td>
<td>Caspase 3</td>
</tr>
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<td>NM_015733</td>
<td>Casp9</td>
<td>Caspase 9</td>
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<tr>
<td>NM_009894</td>
<td>Cideb</td>
<td>Cell death-inducing DNA fragmentation factor, alpha subunit-like effector B</td>
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<tr>
<td>NM_009950</td>
<td>Cradd</td>
<td>CASP2 and RIPK1 domain containing adaptor with death domain</td>
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<td>NM_029653</td>
<td>Dap1</td>
<td>Death associated protein kinase 1</td>
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<td>NM_010044</td>
<td>Dffa</td>
<td>DNA fragmentation factor, alpha subunit</td>
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<tr>
<td>NM_010175</td>
<td>Fadd</td>
<td>Fas (TNFRSF6)-associated via death domain</td>
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<td>Gadd45a</td>
<td>Growth arrest and DNA-damage-inducible 45 alpha</td>
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<td>NM_007545</td>
<td>Hrk</td>
<td>Harakiri, BCL-2 interacting protein (contains only BH3 domain)</td>
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<td>NM_033042</td>
<td>Tnfrsf25</td>
<td>Tumor necrosis factor receptor superfamily, member 25</td>
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<td>NM_011640</td>
<td>Trp53</td>
<td>Transformation related protein 53</td>
</tr>
</tbody>
</table>

**Gene expression using qRT-PCR**

Total RNA was extracted from HCT-116 cells using a RNAqueous Kit (Ambion, Grand Island, NY) and DNase treated. The cDNA was synthesized from 2 μg total RNA using random hexamers and oligo dT primers with SuperScript II reverse transcriptase (Invitrogen). Transcript levels were determined using Taqman gene expression assays.
(Applied Biosystems, Foster City, CA; Cideb Hs00205339_m1, Dapkl
Hs00234489_m1, and Tnfrsf25 Hs00980365_g1) with an ABI Prism 7900HT PCR
sequence detector. Expression levels were normalized to Eukaryotic 18S rRNA
expression (Hs99999901_s1). Linearity of each assay was assessed prior to analysis of
the samples. Negative controls that were prepared during the reverse transcription
reactions by eliminating the RT enzyme were analyzed for each gene.

**Histone H3 and H4 acetylation**

The acetylation levels of histone H3 and H4 were assessed using the EpiQuik
total histone H3/H4 acetylation detection fast kit (Epigentek). Histone proteins were
extracted as per Kit (Epigentek) instructions and the sample concentrations were
measured using a BCA Protein Assay Kit (Pierce, Waltham, MA). Separate assays were
performed to determine acetylation status for the H3 and H4 histones. These assays used
2 μg of nuclear proteins incubated with anti-acetylated histone H3-specific or H4-
specific antibodies. In each assay a detection antibody was added along with a color
development reagent, and the absorbance was measured at 450 nm (Appendix B-16 and
B-17).

**Statistical analysis**

Data were analyzed using one-way ANOVA and Duncan’s multiple range tests
for global methylation, apoptosis, transcription level of Dapkl and Tnfrsf2, and global
H3 or H4 histone acetylation. To determine if gene-specific methylation was different
between each treatment group and the negative control group, the statistical analysis was
carried out using a Wilcoxon rank test. Data are presented as means ± SEM, and means were considered different when the resulting p-value was less than 0.05.

Results

Global methylation

We determined the effects of the PUFA and/or butyrate on the level of global DNA methylation in HCT-116 cells. Both DHA and butyrate independently decreased (p<0.05) global methylation whereas the combination of DHA and butyrate did not produce a significant demethylating effect compared to the negative control (media only). In comparison, the positive control, 5-Aza-dC (2 μM), significantly suppressed global methylation, as expected (Figure 11).

Gene-specific promoter methylation of apoptosis-related genes

We examined DNA methylation status at promoter CpG islands of 24 apoptosis-related genes (Table 10). Of the 24 apoptosis-related genes on the assay card, over 60% of CpG dinucleotides in the promoter of 6 genes (Bcl-2, Bcl2l11, Cideb, Dapkl, Tnfrsf25, and Ltrb) were methylated in the negative control group. The positive control treatment, 5-Aza-dC, induced demethylation only when the CpG rich region of a gene promoter was heavily methylated (> 60%).

Treatment with 5-Aza-dC resulted in a 28% decrease in the level of promoter methylation in Dapkl, compared to the negative control (media only). The promoter methylation level of Bcl2l11 was 97% inhibited by 5-Aza-dC compared with the
Figure 11. Global DNA methylation status (percent of the negative control) in HCT-116 cells cultured with 5-Aza-dC, butyrate, DHA, LA, or co-treatment. Values are means ± SEM. Values not sharing common letters are significantly different, p < 0.05, n = 6 per treatment from 2 independent experiments. Negative controls represent cells incubated in media only. DHA, decosahexaenoic acid; LA, linoleic acid. Data for this figure is found in Appendix Table A-3 and A-5.

Butyrate had demethylating effects on the Bcl2l11 promoter, and DHA treatment significantly decreased the levels of promoter methylation of Cideb, Dapk1, and Tnfrsf25 compared to the negative control group. LA suppressed promoter methylation of Bcl-2, which is an anti-apoptotic mediator. The combination of DHA and butyrate resulted in the demethylation of Bcl2l11, Cideb, Dapk1, Ltbr, and Tnfrsf25.

Colonocyte apoptosis

We next determined if differences found in global methylation and gene-specific methylation affected apoptosis induction. DHA combined with butyrate induced significantly higher levels of apoptosis compared to all other treatment combinations.
(Figure 12). The 5-Aza-dC treatment induced 68% more apoptosis compared to the untreated negative control treatment. Butyrate either by itself or when combined with DHA or LA enhanced apoptosis. The combination of LA and butyrate exhibited a similar level of apoptosis compared to butyrate alone, suggesting that this combination did not augment apoptosis above that obtained with only butyrate.

Figure 12. Colonocyte apoptosis in HCT-116 cells cultured with 5-Aza-dC, butyrate, DHA, LA, or co-treatment (percent of the negative control). Values are means ± SEM. Values not sharing common letters are significantly different, P<0.05, n = 6 per treatment from 2 independent experiments. The negative controls were cells incubated in media only. Data for this figure is found in Appendix Table A-3 and A-5.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Negative control</th>
<th>Positive control 5-Aza-dC</th>
<th>Butyrate</th>
<th>DHA</th>
<th>LA</th>
<th>DHA + butyrate</th>
<th>LA + butyrate</th>
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<td><strong>Highly methylated genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Bcl-2</td>
<td>98.83 ± 0.27</td>
<td>80.62 ± 4.02*</td>
<td>98.26 ± 0.36</td>
<td>94.55 ± 3.31</td>
<td>97.78 ± 0.25*</td>
<td>97.87 ± 0.48</td>
<td>98.35 ± 0.35</td>
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<td>Bcl2l11</td>
<td>68.86 ± 1.74</td>
<td>2.10 ± 0.63*</td>
<td>52.58 ± 10.24*</td>
<td>53.79 ± 10.84</td>
<td>62.22 ± 2.66</td>
<td>52.11 ± 9.82*</td>
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<td>Cideb</td>
<td>94.63 ± 1.52</td>
<td>77.01 ± 2.81*</td>
<td>88.83 ± 2.58</td>
<td>84.69 ± 3.27*</td>
<td>85.02 ± 0.94*</td>
<td>86.89 ± 2.00*</td>
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<td>Dasp1</td>
<td>96.35 ± 0.28</td>
<td>69.84 ± 2.90*</td>
<td>94.86 ± 0.67</td>
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<td>94.88 ± 0.16*</td>
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<td>Ltbr</td>
<td>64.55 ± 1.29</td>
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<td>61.64 ± 1.21</td>
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<td>9.84 ± 2.91</td>
<td>24.80 ± 9.70*</td>
<td>30.42 ± 9.85*</td>
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<tr>
<td>Birc2</td>
<td>2.00 ± 0.17</td>
<td>3.67 ± 1.47</td>
<td>4.62 ± 1.06</td>
<td>4.92 ± 1.36</td>
<td>5.16 ± 1.05*</td>
<td>4.16 ± 0.93*</td>
<td>3.17 ± 1.15</td>
</tr>
<tr>
<td>Bnip3l</td>
<td>0.81 ± 0.10</td>
<td>0.69 ± 0.15</td>
<td>1.03 ± 0.39</td>
<td>1.12 ± 0.21</td>
<td>1.07 ± 0.11</td>
<td>1.03 ± 0.24</td>
<td>0.61 ± 0.19</td>
</tr>
<tr>
<td>Casp3</td>
<td>1.21 ± 0.05</td>
<td>2.06 ± 0.67</td>
<td>10.74 ± 8.13*</td>
<td>2.38 ± 0.61</td>
<td>2.31 ± 0.40*</td>
<td>2.00 ± 0.35*</td>
<td>1.71 ± 0.54</td>
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<tr>
<td>Casp9</td>
<td>3.58 ± 0.34</td>
<td>5.11 ± 0.31*</td>
<td>3.22 ± 0.19</td>
<td>3.44 ± 0.24</td>
<td>3.15 ± 0.26</td>
<td>3.74 ± 0.62</td>
<td>2.80 ± 0.10</td>
</tr>
<tr>
<td>Cradd</td>
<td>1.54 ± 0.56</td>
<td>1.33 ± 0.23</td>
<td>1.48 ± 0.32</td>
<td>1.59 ± 0.37</td>
<td>1.35 ± 0.18</td>
<td>1.69 ± 0.34</td>
<td>1.00 ± 0.20</td>
</tr>
<tr>
<td>Dffa</td>
<td>0.80 ± 0.05</td>
<td>0.65 ± 0.14</td>
<td>0.68 ± 0.25</td>
<td>0.77 ± 0.16</td>
<td>0.74 ± 0.19</td>
<td>0.79 ± 0.19</td>
<td>0.45 ± 0.09*</td>
</tr>
<tr>
<td>Fadd</td>
<td>0.50 ± 0.09</td>
<td>0.50 ± 0.10</td>
<td>0.49 ± 0.19</td>
<td>0.71 ± 0.18</td>
<td>0.67 ± 0.17</td>
<td>0.59 ± 0.12</td>
<td>0.28 ± 0.08</td>
</tr>
<tr>
<td>Gadd45a</td>
<td>10.85 ± 1.26</td>
<td>17.73 ± 6.69</td>
<td>27.02 ± 7.71</td>
<td>32.17 ± 10.33</td>
<td>31.76 ± 5.09*</td>
<td>26.42 ± 6.85</td>
<td>22.36 ± 7.30</td>
</tr>
<tr>
<td>Hrk</td>
<td>16.14 ± 1.23</td>
<td>32.76 ± 4.88*</td>
<td>32.95 ± 6.15</td>
<td>37.31 ± 6.85*</td>
<td>46.33 ± 4.10*</td>
<td>33.85 ± 7.14</td>
<td>30.83 ± 6.23*</td>
</tr>
<tr>
<td>Tnfrsf21</td>
<td>0.67 ± 0.08</td>
<td>0.58 ± 0.19</td>
<td>0.67 ± 0.24</td>
<td>0.79 ± 0.23</td>
<td>0.48 ± 0.10</td>
<td>0.62 ± 0.22</td>
<td>0.37 ± 0.08*</td>
</tr>
<tr>
<td>Trp53</td>
<td>1.20 ± 0.50</td>
<td>1.75 ± 0.67</td>
<td>1.92 ± 0.47</td>
<td>2.11 ± 0.47</td>
<td>2.01 ± 0.39*</td>
<td>1.88 ± 0.33</td>
<td>1.49 ± 0.54</td>
</tr>
</tbody>
</table>

* Data are presented as a percentage of cellular DNA containing methylated gene promoters, which included a combination of both the intermediate level of methylation and hypermethylated DNA.

* Different from the negative control group (p < 0.05). Data are expressed as means ± SEM from 5-6 samples per treatment from 2 independent experiments. The negative controls represent cells incubated in media only.
**The transcription levels of Tnfrsf25 and Dapk1**

To determine the time-course of gene expression, HCT-116 cells were incubated with experimental agents for 48, 72, and 96 h and the transcription levels of *Cideb*, *Dapk1*, and *Tnfrsf25* were quantified by qRT-PCR. These genes were chosen because of their hypermethylated status and response to 5-Aza-dC, DHA, and the combination of DHA and butyrate. The 5-Aza-dC treatment induced the expression of *Dapk1*, and *Tnfrsf25* after 72 and 96 h incubation (Figure 13). *Dapk1* expression was elevated by more than 100 and 200% at 72 h and 96 h of 5-Aza-dC treatment, respectively, as compared with the negative control cells. The transcript level of *Cideb* was only detectable following 5-Aza-dC treatment, suggesting that 5-Aza-dC restored the expression of this gene (data not shown). Butyrate dramatically up-regulated *Tnfrsf25* and *Dapk1* transcript levels at 48h, but declined thereafter. Similar to the data obtained with butyrate alone, the combination of butyrate with DHA or LA resulted in a rapid elevation in transcript levels, followed by a subsequent decline in expression.

**Global histone H3/H4 acetylation**

The increase in transcript levels of *Tnfrsf25* and *Dapk1* by butyrate treatment led us to examine whether gene expression was influenced by a treatment effect on histone acetylation status. Therefore, we assessed the global histone acetylation levels of H3 and H4 in HCT-116 cells. Cells treated with 5-Aza-dC demonstrated no significant changes in histone acetylation state. Interestingly, global H3 histone acetylation levels were elevated in butyrate treated cells compared to the negative controls, but there was no significant difference between butyrate and the other treatment groups. The global H4
Figure 13. Time-course analysis of Tnfrsf25 (A) and Dapk1 (B) transcript levels in HCT-116 cells cultured with 5-Aza-dC, butyrate, DHA, LA, or co-treatment (expressed as percentage of the negative control). Values are means ± SEM. *Different from the negative control sample measured at the same time point, \( P < 0.05 \), \( n = 3 \) per treatment. The negative controls were cells incubated in media only. Data for this figure is found in Appendix Table A-4 and A-6.
histone acetylation level increased in cells treated with LA or LA combined with butyrate (Figure 14).

![Graph showing histone acetylation levels in HCT-116 cells.](image)

Figure 14. H3 and H4 histone acetylation in HCT-116 cells incubated with 5-Aza-dC, butyrate, DHA, LA, or co-treatment (expressed as percentage of the negative control). Values are means ± SEM. *Different from the negative control, P < 0.05, n = 3 per treatment. The negative controls were cells incubated in media only. Data for this figure is found in Appendix Table A-3 and A-5.

**Discussion**

The transcriptional regulation of apoptosis-associated genes by DNA methylation is one of the mechanisms by which cancerous cells avoid apoptosis. The aberrant alteration of DNA methylation is either by hypermethylation to suppress expression of
proapoptotic genes or by hypomethylation to promote expression of antiapoptotic genes (43).

Studies have been conducted on DNA methylation status of apoptosis-related genes in prostate cancer (157), glioblastoma multiforme (158), and bladder cancer (44). Specifically, promoter regions of apoptosis-related genes are hypermethylated and correlate with tumor phenotype, so that aberrant DNA methylation of apoptosis-related genes can be considered a biomarker for cancer diagnosis.

In this study, we investigated the promoter methylation status of 24 apoptosis-related genes in response to DHA or LA and/or butyrate co-treatment in human colon cancer cells. Methylation of apoptosis-related genes was targeted because we previously demonstrated that the combination of fish oil/n-3 PUFA and pectin/butyrate affected the expression level of genes involved in apoptosis and enhanced colonocyte apoptosis (85, 139). The goal of this paper was to determine if DHA and/or butyrate modulates the expression level of apoptosis-related genes by suppressing promoter methylation in vitro. Hypermethylation was detected for six apoptosis-related genes, including Bcl-2, Bcl2l11, Cideb, Dapk1, Libr, and Tnfrsf25, which are also methylated in colon cancer or other types of cancers (45, 47, 159-161). Friedrich et al. had similar results in bladder tumor samples, wherein Dapk1, and Tnfrsf25 were hypermethylated compared with nonmalignant adjacent tissue (44).

DHA and LA treatments decreased promoter methylation of Cideb, Dapk1, and Tnfrsf25, which are all linked to a proapoptotic phenotype. However, LA combined with butyrate did not exhibit a demethylating effect on these three genes. DHA combined
with butyrate also reduced DNA methylation of these three genes as well as \textit{Bcl2l11} and \textit{Ltbr} (Table 10). We have previously demonstrated that combination chemotherapy using DHA and butyrate most effectively enhanced colonocyte apoptosis (96). We demonstrate for the first time that part of the enhanced apoptosis by the combination of DHA and butyrate may be attributable to the demethylating effect of proapoptotic genes in colon cancer cells.

Butyrate decreased promoter methylation of \textit{Bcl2l11} as well as global methylation. In addition, it caused a 4-fold increase in the transcription levels of \textit{Tnfrsf25} and \textit{Dapk1} at 48 h, and also increased their expression when combined with LA and DHA. These increases in gene expression might be due to an effect of butyrate on histone deacetylation because it is a well-known inhibitor of histone deacetylase. It is known that histone acetylation mainly occurs at the promoter region of genes in the process of transcription whereas histone deacetylation occurs in the promoter region leading to gene expression silencing (162). The promoter region of \textit{Dapk1} and \textit{Tnfrsf25} are proximate to sites where histone modifications occur (163). Therefore, the increased histone acetylation by butyrate might contribute to the induction of gene expression of these genes. We found that butyrate enhanced global histone H3 methylation and this result is comparable to that of Kobori et al., who noted that 10 mM butyrate enhanced 25\% more histone H3 acetylation than a control group in human colon cancer cells (164). However, it will be necessary to study site-specific histone acetylation to fully appreciate the influence of histone modifications on increases of \textit{Dapk1} and \textit{Tnfrsf25} expression.
With respect to the time-dependent increases in gene expression due to the 5-Aza-dC treatment, our results are comparable to those of others showing that 5-aza-cytidine, the analog of 5-Aza-dC, increased the expression level of p16 as incubation time progressed (165). Indeed, 72 h exposure to 2 µM 5-Aza-dC suppressed \(Dapk1\) promoter methylation by 28% and induced a 1.5-fold increase in \(Dapk1\) transcription (Figure 13). Recently, Shu et al. reported that curcumin resulted in a similar reduction in promoter methylation of \(Neurog1\) in prostate cancer cells and that this reduction led to the 2-fold increase of \(Neurog1\) mRNA expression (71). However, the decreases in promoter methylation caused by DHA, compared to the negative control in this study were less than 50% of the decrease obtained with the positive control (5-Aza-dC, 18-27%).

The 5-Aza-dC treatment, a DNMT inhibitor, has been intensively studied as a compound that inhibits DNA methylation and restores the expression level of tumor suppressor genes (38). This compound is incorporated into DNA and acts as a substrate for DNMT. However it adheres to DNMT and remains as a covalent protein-DNA adduct, thereby suppressing DNA methylation. Dietary compounds, such as epigallocatechin-3-gallate (EGCG, green tea compound) interrupt DNMT activity by blocking the active site of the enzyme (65). Therefore, it is possible that the demethylating effect of DHA might be exerted through mechanisms other than that employed by 5-Aza-dC. DHA is primarily incorporated into phosphatidylethanolamine (PE), which is converted to phosphatidylcholine (PC) through the addition of methyl groups from S-adenosyl methionine (79). Recently, Kale et al. found that when DHA
was lacking, there was less PE-containing DHA, and the resulting excess in available methyl groups could be used for DNA methylation by DNMT (77). This finding suggests that DHA could suppress DNA methylation by consuming methyl groups. Therefore, DHA may indirectly suppress DNMT activity whereas 5-Aza-dC directly inactivates DNMT. In our current study, DHA inhibited global methylation and promoter methylation of proapoptotic genes, yet the demethylating effect of DHA was not sufficient to induce gene expression. There was a similar report that reduced promoter methylation by butyrate was not sufficient to allow re-expression of genes (166).

In summary, we have shown that the combination of DHA and butyrate promotes apoptosis in part by enhancing promoter methylation of the proapoptotic genes Bcl2l11, Cideb, Dapk1, Ltbr, and Tnfrsf25 in colon cancer cells. We observed that DHA has global and modest gene-specific demethylating activity, which may have implications for a synergistic effect with the inhibitor of DNA methylation, such as 5-Aza-dC.
CHAPTER V
SUMMARY AND CONCLUSION

Summary

Colon cancer is the second leading cause of cancer mortality in the United States. The incidence of colon cancer is known to be affected by environmental factors, such as diet. Studies using AOM injected rats revealed that the combination of FO/P, compared to CO/C, is protective against colon cancer (95). Fish oil contains high concentrations of n-3 PUFA, such as DHA and EPA which are bioactive compounds in fish oil. In contrast, corn oil contains high levels of n-6 PUFA like linoleic acid that is prevalent in Western diets. It is documented that diets containing high levels of n-6 PUFA promote colon cancer (84). Fermentable fibers, such as pectin, can be utilized by microorganisms in the colon and fermented to short chain fatty acids like butyrate. One mechanism whereby the combination of FO/P is chemoprotective is the induction of apoptosis, a programmed form of cell death (95, 98).

Accumulated genetic mutations of tumor suppressor genes and oncogenes are found during colon cancer development. If DNA mutations occur within sequences encoding proteins, it could disrupt the expression and function of those genes, resulting in the growth of neoplastic cells. In addition, epigenetic alterations, such as DNA methylation and histone modification, contribute to colon carcinogenesis through changes in gene transcription. The epigenetic regulation of transcription is
accomplished by preventing transcription factor binding or providing a binding site for methyl-binding proteins, which form a repressive complexes with histone deacetylases and histone methyltransferases.

To monitor the changes in the expression of genes involved in apoptosis and cell proliferation during the tumorigenic process, we analyzed temporal gene expression profiles from exfoliated rat colonocytes. The combination of FO/P modulated global gene expression profiles at biologically important time points in carcinogen injected rats and these changes in gene expression were consistent with phenotypic observations at each time point. At initiation, FO/P down-regulated the expression of genes involved with cell adhesion and enhanced apoptosis compared with CO/C. At the ACF stage, the expression of genes involved in cell cycle regulation was modulated by FO/P and the zone of proliferation was reduced in FO/P rats compared with CO/C rats. The diet containing FO/P also increased apoptosis and the expression of genes that promote apoptosis at the tumor endpoint compared with CO/C.

We then proceeded to determine if changes in expression of genes involved in apoptosis in response to a FO/P diet are associated with changes in methylation of the promoter regions of these genes. Methylation of the Bcl-2 promoter was targeted because we previously demonstrated that the combination of FO/P, compared with CO/C, suppressed the level of Bcl-2 expression and the induction of colonocyte apoptosis (139). To test the hypothesis that changes in expression of Bcl-2 in response to a FO/P diet are associated with changes in methylation of the promoter region of Bcl-2, the methylation status of the Bcl-2 promoter region and the transcription level of Bcl-2
were measured in colon tumors and non-involved colon tissues of rats exposed to a carcinogen. The FO/P diet increased \textit{Bcl-2} promoter methylation in tumor tissues and colonocyte apoptosis relative to those observed with the CO/C diet. A tendency for a negative relationship between \textit{Bcl-2} DNA methylation and \textit{Bcl-2} mRNA levels was observed in the tumors.

The final study examined the ability of DHA and butyrate to affect DNA methylation of genes involved in apoptosis using human colon cancer cells. The combination of DHA and butyrate led to a significant reduction in the methylation of \textit{Bcl2l11}, \textit{Cideb}, \textit{Dapk1}, \textit{Ltbr}, and \textit{Tnfrsf25}, which are all pro-apoptotic genes that exhibit highly methylated promoter regions in these cancer cells. The combination of DHA and butyrate also led to an increase in apoptosis in this colon cancer cell line. DHA treatment provoked global demethylation and the decreases in the methylation of \textit{Cideb}, \textit{Dapk1}, and \textit{Tnfrsf25}, whereas LA suppressed promoter methylation of \textit{Bcl-2}.

\textbf{Conclusion}

The effects of chemotherapeutic diets on epithelial cell gene expression can be monitored noninvasively throughout the tumorigenic process. This noninvasive technique makes it feasible to monitor human disease progression and identify critical time points for potential diet intervention. The diet containing FO/P is chemoprotective in part due to its ability to affect expression of genes involved in apoptosis and cell cycle regulation throughout all stages of tumorigenesis. The changes in the transcription
levels of apoptosis-related genes and the induction of apoptosis by this dietary intervention were regulated through changes in the methylation state of these genes. Taken together, this study enhances an understanding of how the chemoprotective FO/P diet modulates the transcription of apoptosis-related genes during colon carcinogenesis, so that we could have an underlying principle for the benefits of this dietary intervention on colon cancer.

**Future research**

This was the first study to show that a chemoprotective FO/P diet modulates epigenetic mechanisms including promoter methylation. We saw significant effects of FO/P on promoter methylation of apoptosis-related genes in human colon cancer cells and a carcinogen injected colon cancer model, but further research is needed.

Future research should study the mechanism by which FO/P modulates promoter methylation of cancer-related genes. This work should determine if the combination of n-3 PUFA and butyrate are affecting DNA methylation through their ability to influence either expression or activity of DNA methyltransferases (DNMTs), which are the enzymes that regulate methyl group transfer. It has been reported that DNMT1 is over-expressed in several types of cancers. Experiments could be planned to investigate the dose and time dependent effects of n-3 PUFA and/or butyrate on the activity of DNMT1, DNMT3A and DNMT3B in normal and colon cancer cell lines.
It would be also beneficial to study the effect of DHA on the supply of S-adenosyl methionine. Dietary DHA could increase DHA-containing phospholipids which competitively use methyl groups. Future experiments should determine if DHA affects the availability of the methyl group which can be used for DNA methylation.
LITERATURE CITED


90. Siavoshian S, Blottiere HM, Le Foll E, Kaeffer B, Cherbut C, Galmiche JP. Comparison of the effect of different short chain fatty acids on the growth and


apoptosis in part through suppression of PPARdelta/PGE2 and elevation of PGE3.


APPENDIX A

TABLES OF RESULTS

Chapter III
Table A-1. The effect of FO/P on colon cancer incidence, apoptosis index, Bcl-2 promoter methylation, and Bcl-2 expression

<table>
<thead>
<tr>
<th>Item</th>
<th>CO/C</th>
<th>FO/P</th>
<th>P-value (one-sided)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon tumor incidence (%)</td>
<td>75.60</td>
<td>51.50</td>
<td>0.016</td>
</tr>
<tr>
<td>Apoptosis index</td>
<td>1.40 ± 0.23</td>
<td>2.62 ± 0.52</td>
<td>0.020</td>
</tr>
<tr>
<td>Relative Bcl-2 promoter methylation at 16 wk (non-involved tissues) (%)</td>
<td>100.00 ± 16.54</td>
<td>68.64 ± 12.87</td>
<td>0.083</td>
</tr>
<tr>
<td>Relative Bcl-2 promoter methylation at 34 wk (non-involved tissues) (%)</td>
<td>100.00 ± 10.72</td>
<td>134.48 ± 29.47</td>
<td>0.204</td>
</tr>
<tr>
<td>Relative Bcl-2 promoter methylation at 34 wk (carcinomas) (%)</td>
<td>153.27 ± 17.54</td>
<td>245.11 ± 50.31</td>
<td>0.009</td>
</tr>
<tr>
<td>Relative Bcl-2 gene expression in carcinomas (%)</td>
<td>100.00 ± 31.74</td>
<td>53.32 ± 21.52</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Values are means ± SEM.

Chapter III
Table A-2. The effect of time on Bcl-2 promoter methylation

<table>
<thead>
<tr>
<th>Item</th>
<th>16 wk</th>
<th>34 wk</th>
<th>P-value (one-sided)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative Bcl-2 promoter methylation (non-involved tissues) (%)</td>
<td>100.00 ± 13.11</td>
<td>52.87 ± 6.18</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Values are means ± SEM.
Chapter IV

Table A-3. Global methylation, apoptosis, and histone acetylation in HCT-116 cells cultured with 5-Aza-dC, butyrate, DHA, LA, or co-treatment (% of negative control)

<table>
<thead>
<tr>
<th></th>
<th>Negative control</th>
<th>Positive control 5-Aza-dC</th>
<th>Butyrate</th>
<th>DHA</th>
<th>LA</th>
<th>DHA + butyrate</th>
<th>LA + butyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Global methylation</strong></td>
<td>100.00 ± 5.86</td>
<td>60.63 ± 5.52</td>
<td>68.39 ± 11.38</td>
<td>65.85 ± 11.50</td>
<td>89.32 ± 12.17</td>
<td>88.35 ± 9.48</td>
<td>89.78 ± 4.62</td>
</tr>
<tr>
<td><strong>Colonocyte apoptosis</strong></td>
<td>100.00 ± 7.42</td>
<td>167.87 ± 11.73</td>
<td>177.05 ± 27.44</td>
<td>68.59 ± 11.38</td>
<td>95.41 ± 15.65</td>
<td>284.31 ± 64.94</td>
<td>169.67 ± 36.28</td>
</tr>
<tr>
<td><strong>Histone acetylation</strong></td>
<td>H3 100.00</td>
<td>93.58 ± 5.91</td>
<td>119.95 ± 5.36</td>
<td>120.74 ± 0.62</td>
<td>123.01 ± 0.77</td>
<td>122.18 ± 3.95</td>
<td>116.98 ± 3.11</td>
</tr>
<tr>
<td></td>
<td>H4 100.00</td>
<td>83.76 ± 14.72</td>
<td>115.51 ± 6.36</td>
<td>103.47 ± 13.11</td>
<td>137.59 ± 13.94</td>
<td>116.06 ± 22.92</td>
<td>177.92 ± 12.00</td>
</tr>
</tbody>
</table>

Values are means ± SEM.

Chapter IV

Table A-4. Tnfrsf25 and Dapk1 expression in HCT-116 cells cultured with 5-Aza-dC, butyrate, DHA, LA, or co-treatment (% of negative control)

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Time</th>
<th>Negative control</th>
<th>Positive control 5-Aza-dC</th>
<th>Butyrate</th>
<th>DHA</th>
<th>LA</th>
<th>DHA + butyrate</th>
<th>LA + butyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tnfrsf25</td>
<td>48h</td>
<td>100.00 ± 11.15</td>
<td>103.60 ± 26.54</td>
<td>519.94 ± 84.70</td>
<td>79.95 ± 16.31</td>
<td>57.54 ± 20.02</td>
<td>386.18 ± 115.62</td>
<td>234.98 ± 43.78</td>
</tr>
<tr>
<td></td>
<td>72h</td>
<td>100.00 ± 17.70</td>
<td>153.18 ± 7.76</td>
<td>88.63 ± 14.39</td>
<td>67.11 ± 25.36</td>
<td>75.92 ± 6.72</td>
<td>65.20 ± 15.15</td>
<td>85.48 ± 18.18</td>
</tr>
<tr>
<td></td>
<td>96h</td>
<td>100.00 ± 6.10</td>
<td>141.20 ± 11.06</td>
<td>45.74 ± 7.87</td>
<td>109.70 ± 7.07</td>
<td>97.11 ± 3.86</td>
<td>35.62 ± 3.26</td>
<td>37.48 ± 5.52</td>
</tr>
<tr>
<td>Dapk1</td>
<td>48h</td>
<td>100.00 ± 9.08</td>
<td>137.31 ± 33.81</td>
<td>500.57 ± 103.09</td>
<td>57.07 ± 2.03</td>
<td>68.78 ± 6.58</td>
<td>281.22 ± 55.93</td>
<td>216.67 ± 16.79</td>
</tr>
<tr>
<td></td>
<td>72h</td>
<td>100.00 ± 9.57</td>
<td>244.54 ± 10.93</td>
<td>168.91 ± 31.67</td>
<td>73.05 ± 12.71</td>
<td>71.32 ± 4.86</td>
<td>106.80 ± 31.61</td>
<td>149.99 ± 21.43</td>
</tr>
<tr>
<td></td>
<td>96h</td>
<td>100.00 ± 3.32</td>
<td>319.42 ± 7.89</td>
<td>35.29 ± 6.75</td>
<td>78.58 ± 6.64</td>
<td>77.33 ± 1.62</td>
<td>25.40 ± 5.65</td>
<td>26.22 ± 2.24</td>
</tr>
</tbody>
</table>

Values are means ± SEM.
## Chapter IV

### Table A-5. P-values for global methylation, apoptosis, and histone acetylation in HCT-116 cells cultured with 5-Aza-dC, butyrate, DHA, LA, or co-treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Apoptosis index</th>
<th>Global methylation</th>
<th>Histone H3 acetylation</th>
<th>Histone H4 acetylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-Aza-dC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butyrate</td>
<td>9.04E-02</td>
<td>1.81E-02</td>
<td>1.49E-03</td>
<td>4.33E-01</td>
</tr>
<tr>
<td>DHA</td>
<td>4.83E-01</td>
<td>1.10E-02</td>
<td>1.09E-03</td>
<td>8.59E-01</td>
</tr>
<tr>
<td>LA</td>
<td>9.18E-01</td>
<td>3.99E-01</td>
<td>4.62E-04</td>
<td>7.08E-02</td>
</tr>
<tr>
<td>DHA + Butyrate</td>
<td>1.93E-04</td>
<td>3.58E-01</td>
<td>6.31E-04</td>
<td>4.17E-01</td>
</tr>
<tr>
<td>LA + Butyrate</td>
<td>1.24E-01</td>
<td>4.34E-01</td>
<td>4.76E-03</td>
<td>1.18E-03</td>
</tr>
<tr>
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- Positive control
- Negative control
- 5-Aza-dC
- Butyrate
- DHA
- LA
- DHA + Butyrate
- LA + Butyrate

**Histone acetylation**
- H3
- H4
Table A-6. P-values for Tnfrsf25 and Dapk1 expression in HCT-116 cells cultured with 5-Aza-dC, butyrate, DHA, LA, or co-treatment

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</tbody>
</table>
APPENDIX B

EXPERIMENTAL PROTOCOLS

B-1. Sample Preparation for qRT-PCR

Purpose: To prepare RNA samples for gene expression measurement

Procedure

1. RNA isolation using RNAqueous kit (Ambion, AM1912); small scale phenol-free total RNA isolation kit without modification.

2. DNase treatment using DNA-free kit (Ambion, AM1906)
   See Appendix B-2.

3. Quantification using Nanodrop

4. Agilent analyzer to check the quality of RNA
   Select a kit depending on concentration of RNA samples.
   - Total RNA and mRNA samples of 25 to 500 ng/µl
     ⇒ Agilent RNA 6000 Nano Kit (Cat. # 5067-1511 from Agilent Technologies)
   - For samples down to 50 pg/µl of total RNA or 250 pg/µl of mRNA.
     ⇒ Agilent RNA 6000 Pico Kit (Cat. # 5067-1513 from Agilent Technologies)

5. Reverse transcription to make cDNA
   See Appendix B-3.

6. Run qRT-PCR
B-2. DNase treatment using DNA-free kit (Ambion, AM1906)

Purpose: To remove contaminating DNA from RNA preparations

Procedure

1. Add 10X DNase I Buffer (0.1 volume of RNA sample) and 1 μl rDNase I to the RNA, and mix gently by flicking the tube and spin down using countertop centrifuge. (If the RNA contains more than 20 ng/μl, dilute the sample to 20 ng/μl before adding the DNase I Buffer and rDNase I)

2. Incubate at 37°C for 30 min.

3. Add resuspended DNase Inactivation Reagent (typically 0.1 volume of sample from step #1) and mix well.

4. Incubate 2 min at room temperature, mixing occasionally.

5. Centrifuge at 10,000 x g for 1.5 min.

6. Transfer the RNA (in supernatant) to a new tube.
B-3. Reverse Transcription

Purpose: To prepare cDNA from RNA for PCR

Gibco SuperScript II RT Protocol
Cat # 18064-014
Use ART Tips and Nuclease free water.

25 µl reaction
Random hexamers   0.24 µl Roche 11034731001 (1 mM stock)
Oligo dT (500 ng/µl)   1.25 µl Promega Oligo(dT)15 primer
RNA     0.2-200 ng
Nuclease free water   up to 14.75 µl

Heat above components at 65 °C, 5 min using Thermocycler (file name; YMC65RT). Cool slowly to room temperature. (5°C decrease every two minutes)

Make a cocktail:
5X 1st strand buffer  5 µl Invitrogen (18064-014)
0.1M DTT   2.5 µl Invitrogen (18064-014)
*Primer RNase inhibitor  0.5 µl Eppendorf
10 mM dNTP mix 1.25 µl Promega -10 mM each dNTP
SuperScript® II RT   1 µl Invitrogen (18064-014)

Add 10.25 µl of cocktail Mix. Mix each tube and spin down using countertop microfuge. Incubate at 37 °C, 1 hr. Terminate by heating at 90 °C, 5 min. Place on ice and freeze at -80 °C.

* 7500 unit, 30 Unit/µl, recombinant, not human placental

The negative RT is done by replacing the SuperScript with Nuclease free water.
B-4. Apoptosis – ApopTag

Purpose: To determine apoptosis in 4% PFA fixed colon tissue.

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<th>reagent</th>
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<th>catalog #</th>
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</thead>
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<td>Proteinase K</td>
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</tr>
<tr>
<td>PBS</td>
<td>Life Technologies</td>
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</table>

Note: To be performed on 4% PFA fixed tissue.

***Put 200 ml PBS for Prot. K in 37° C oven and begin bleach rinse.

1. Deparaffinize and rehydrate tissue:
   ___Xylene, 3X, 5 min
   ___let xylene just dry, circle sections w/ PAP pen, dry 1 min
   ___100% EtOH, 2X, 5 min
   ___95% EtOH, 1X, 3 min
   ___70% EtOH, 1X, 3 min
   ___PBS, 1X, 5 min
   (Get Equilibration Buffer and Reaction Buffer out of freezer-put on ice)

2. Pretreat tissue – 3 min, in 37°
   Proteinase K (10 µg/ml PBS) = 0.1 ml Proteinase K (Ambion # 2546) in 200 ml PBS.

3. Wash in dH₂O, 2x, 2 min

4. Quench Endogenous Peroxidase: 0.3% \( \text{H}_2\text{O}_2 \) in 100% Methanol:
   3.0 ml 30% \( \text{H}_2\text{O}_2 \) in 297 ml 100% Methanol or 2.0 ml in 198 ml (add fresh \( \text{H}_2\text{O}_2 \) immediately before quenching). 30 min, RT

5. Wash in dH₂O, 2x, 5 min

6. Wash all slides in PBS 5 min.

7. Gently tap off PBS and carefully blot around sections. (Do this step and following step one slide at a time to avoid drying out sections.)

8. Apply EQUILIBRATION BUFFER to all sections: incubate in humidified chamber for 15 sec to 1 hr @ RT.
   (# of slides X 150 µl) (e.g., 9 slides X 150µl = 1.35 ml)
9. Tap off equilibration buffer and immediately apply REACTION BUFFER (for negative controls) or working strength TdT Enzyme with dilution ratio 1/40 (enzyme/reaction buffer for sample sections). (Get TdT directly from freezer & keep on ice)

Apply only reaction buffer to negative control sections:

___(# sections) X 40µl

For normal sample sections (# sections X 50µl):

___ µl reaction buffer (for ___ slides)
___ µl TdT enzyme (for ___ slides)

Incubate in a humidified chamber at 37°C, 1 hr
(Prepare Stop/Wash so it can warm to RT.)

12. Put slides in coplin jar with Working Strength Stop/Wash Buffer (1ml + 34 ml dH₂O). Agitate for 15 sec; incubate 10 min, RT.
Take aliquot of ANTI-DIGOXIGENIN PEROXIDASE (# slides X 150 µl) and allow to warm to room temperature (___ slides x 150µl = ______)

13. Wash slides in PBS, 3X, 1min

14. Blot dry the slides quickly (do one slide at a time) and apply ANTI-DIGOXIGENIN PEROXIDASE to all sections; incubate 30 min. in humidified chamber @ RT.

15. Wash in PBS 4X, 2min

16. While washing prepare 0.5% DAB solution (0.5 mg/ml):
Filter 2 ml stock DAB (50 mg/ml) through slightly damp (w/PBS) Whatman #1 filter and follow through filter w/198 ml PBS. Also, prepare dH₂O for after staining (step 19).

17. IMMEDIATELY before staining add 100 µl fresh 30% H₂O₂ to DAB solution.

18. Stain slides until light brown color shows up (< 20 sec)

19. Wash in dH₂O, 3X, 1 min
   Leave in 4th wash for 5 min
   During this wash deactivate DAB solution and equipment w/ bleach – leave these materials in bleach overnight.
20. Counterstain w/ Methyl Green (reusable):
   Dip quickly into Methyl green
   Rinse in dH₂O 5X;
   1) Dip and briefly agitate and move to second dH₂O.
   2) Dip and briefly agitate and move to third dH₂O.
   3) Dip 10 times and leave ~ 30 sec.
   4) Leave for 1 min without agitation.
   5) Leave for 1 min without agitation.

21. Dehydrate: USE ALL FRESH REAGENTS
   __70% EtOH, 1X, 1 min
   __95% EtOH, 1X, 1 min
   __100% EtOH, 1X, 1 min
   __Xylene: 3X, 2 min (dip 10 times/ea)

22. Apply Permount and cover glass. (80:20, Permount:Xylene)
Use the wet mounting method (Do one slide at a time. Don’t allow a slide to dry after last xylene.) Dry 24 hours before viewing on microscope.
B-5. Cell proliferative zone using PCNA methodology

Purpose: To determine cell proliferative zone in 70% ethanol fixed colon tissue.

Staining protocol using the sequenza slide rack (Shandon, Pittsburgh, PA)

Reagents:

1. Phosphate buffered saline (PBS)
2. Vectastain ABC Elite Kit -- mouse IgG
   Source: Vector Lab.
   Cat #: PK-6102, 1 ml/ea.
   Contains: blocking serum
   biotinylated antimouse IgG
   avidin-biotin complex
3. PCNA monoclonal antibody (anti-PC10, murine)
   Source: Signet lab
   Cat #: 523-01, 1ml/ea
   Note: Each new lot # of anti-PC10 antibody must be tested with a 1:50, 1:100,
   1:150, 1:200 dilution in PBS. Prepare antibody dilution with PBS, under sterile
   conditions. Use 4 micron thick single serial sections of colonic tissue to test the
   antibody dilutions. Record the optimal dilution for the lot # tested.
4. Diaminobenzidine or DAB-tetrahydrochloride
   Source: Sigma
   Cat #: D-5637
   Prepare DAB stock solution, 50 mg/ml in ddH₂O. Store as 2 ml aliquots at -20°C.
5. Harris hematoxylin solution
   Source: Sigma
   Cat #: HHS16-500ML

Critical steps prior to staining:
1. Tissue fixation in 70% ethanol.
2. Processing and baking of tissue at temperatures not exceeding 50°C.
3. Keep slides moist at all times during the staining procedure.
4. Include one negative control slide (slide not treated with antibody, PBS only) in each
   batch of slides stained.

Procedures:
1. Deparaffinize slides
   Xylene, 3X, 5 min
   100% EtOH, 2X, 5 min
   95% EtOH, 2X, 3 min
   70% EtOH, 1X, 3 min
   ddH₂O, 1X, 3 min
2. Leave slides in 3% H$_2$O$_2$ for 30 min to remove endogenous peroxidase activity.  
   3% H$_2$O$_2$: 20 ml of 30% H$_2$O$_2$ made up to a final volume of 200 ml with methanol.

3. Wash in PBS 5 min, 3 times.  
   Mount slides onto coverplates using PBS and insert into sequenza.

4. Prepare Vectastain Blocking Serum by adding 3 drops of stock Normal Serum (yellow label) to 10 ml of PBS in yellow mixing bottle.

5. Add 3 drops* to each coverplate in sequenza and replace top to maintain humidity.  
   Let stand for 20 min.

6. Wash with PBS by filling each coverplate in the sequenza with one ml of PBS.

7. Add 150 µl of anti-PC10 (use the optimal dilution obtained by dilution series: 1:200) to each coverplate in sequenza and replace top.  For the negative control slide use PBS instead of anti-PC10.  Let stand for 1 hour.

8. Wash with PBS by filling each coverplate in the sequenza to the top with PBS.  Let stand for 5 min.

9. Prepare Vectastain biotinylated anti-mouse IgG by mixing 3 drops of stock normal serum with 10 ml PBS in Blue mixing bottle, then add 1 drop of stock biotinylated antibody (blue label), Mix Gently.  (or use 5 ml of serum solution from step 4, mix with 25 µl of stock biotinylated antibody)

10. Add 3 drops to each coverplate in sequenza and replace top.  Let stand for 45 min.

11. Prepare ABC reagent in advance to be used in step 13.  The ABC reagent is prepared by adding 2 drops of reagent A (gray label) to 5 ml PBS in the ABC Reagent large mixing bottle.  Then add exactly 2 drops of Reagent B (gray label) to the same mixing bottle, mix immediately, and allow ABC Reagent to stand for about 30 minutes before use.  This should be done in a darkened area.

* Note: 1 drop = 50 µl

12. Wash with PBS by filling each coverplate in the sequenza to the top with PBS.  Let stand for 5 min.

13. Apply Vectastain ABC reagent that had been prepared in step 11.  Add 3 drops to each coverplate in sequenza, replace top, and let stand for 30 min.

14. Wash with PBS by filling each coverplate in the sequenza to the top with PBS.  Remove slides from sequenza and place in PBS for 5 min.
15. Prepare DAB solution
   For 200 ml: 2 ml stock DAB (50 mg/ml)
   bring volume up to 200 ml with PBS
   Immediately before staining, add 100:1 30% H₂O₂.

16. Leave slides in DAB solution for 1 min, agitate twice.

17. Wash with ddH₂O 5 min, 3 times.

18. Deactivate all DAB materials (glassware, pipet tips, used stock vials, stir bars) and
    used DAB solutions with bleach overnight, and flush with excess water in drain next
day.

19. Counterstain with Hematoxylin ≤ 1 sec.

20. Wash with ddH₂O 2 min, 2 times.

21. Dehydrate slides:
    1 x 1 min 70% ETOH.
    1 x 1 min 95% ETOH.
    1 x 1 min 100% ETOH.
    1 x 2 min Xylene.

22. Apply Permount and cover glass. (80:20, Permount:Xylene)
    Use the wet mounting method (Do one slide at a time. Don’t allow a slide to dry after
    last xylene.) Dry 24 hours before viewing on microscope.
B-6. Design of PCR primers & probe to determine promoter methylation


Purpose: Design PCR primers and probes to measure promoter methylation using qPCR

Procedure

   Select species. (Rat or Human)
   Input gene name (For example, Bcl-2)
   Results summary shows Domain, Family, Gene, Marker, etc.
   Select “Gene”
   Among the list of genes, select Bcl-2.

2. In the left column, select “sequence”.
   In the bottom of left column, there is “Configure this page”
   Change 600 to 1000 of 5’ flanking sequence (upstream)
   Now we know the sequence of Bcl-2 and the location of the first exon
   (transcription start site) that is highlighted with pink background.
   Copy sequence of gene from the first to the end of the first exon.

3. http://genome.ucsc.edu/
   Select “Blat” in the bar of the main menu.
   Select species.
   Paste the sequence from # 2 step and hit “submit”.
   Select the first “Browser”
   Scroll down to “Expression and regulation”
   CpG islands “pack” activate CpG island ➔ Refresh
   Zoom out X3
4. Select CpG island which is close to the transcription start site. Click the selected CpG island in the figure. “CpG island info” gives us the position, the sequence of the CpG island. Click the “View DNA for this feature.

5. In Microsoft word, paste DNA sequence of CpG island
   1) Replace “CG” → “XG” (All CG have to change to XG)
   2) Replace all “C” → “T”
   3) Replace all “X” → “C”
These steps give us bisulfite modified DNA sequence with the assumption that all CpG were methylated.

6. Bring bisulfite modified DNA sequence of CpG island to the primer design software, Primer express 3.0
   Check the type as TaqMan MGB quantification.
   Check “Double stranded” Box
Click “Find Primers/Probes”

Criteria for primers & probe design
1. Try to choose primers that have CpGs at the 3’end of the sequence and many non-CpG cytosines throughout the primer.
2. For the probe, make sure that the 5’ base is not a G, try to use probe with many non-CpG cytosines throughout the probe and that the CpGs are in the middle of the sequence.
3. Probe should not be more than 31-32 bp in length.
4. Avoid primers and probes with long stretches of single nucleotide repeats. 
   (AAAAAAAA)
5. Design all reactions with a primer Tm from 58-60 °C, and the probe Tm of 68-70 °C.

Let’s start from primers and probe set from primer express 3.0.
Bring “Primer probe test tool” and modify the sequence to get the best primer and probe within criteria. These primers and probe will amplify only the fully methylated DNA.
B-7. Preparation of fully methylated DNA using CpG methyltransferase

Reference:
doi:10.1038/nprot.2006.152

Purpose: To generate artificially fully methylated genomic DNA, which will serve as a positive control.

Materials:
- CpG Methyltransferase (M.SssI) Cat #. M0226L from New England Biolabs
- 10X buffer and S-adenosyl methionine (SAM) were provided with M.SssI.
- DNA clean & concentrator-5. Cat #. D4003S from Zymo research
- Genomic DNA of rat liver extracted using DNeasy Blood & Tissue Kit Cat #. 69504 from Qiagen
- Turn on the incubator 37°C

Procedure

CpG Methyltransferase treatment

1. Dilute SAM to 1.6 mM using the supplied 32 mM SAM stock.
   SAM stock 1 µl + RNase/DNase free water 19 µl
2. Dilute M.SssI to make 0.05 unit M.SssI.
   M.SssI 1 µl (4 units) + RNase/DNase free water 79 µl
3. Add DNA, diluted SAM, 10X buffer, and diluted M.SssI in 1.5 ml microcentrifuge tubes.

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<tbody>
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<td>DNA</td>
<td>0.05 µg/µl</td>
<td>µg/µl</td>
<td>1 µg</td>
</tr>
<tr>
<td>SAM</td>
<td>0.16 mM</td>
<td>mM</td>
<td>2 µl (diluted)</td>
</tr>
<tr>
<td>10X buffer</td>
<td>1 X</td>
<td></td>
<td>2 µl</td>
</tr>
<tr>
<td>Enzyme</td>
<td>0.0025 unit/µl</td>
<td></td>
<td>1 µl (diluted)</td>
</tr>
<tr>
<td>RNase/DNase free water</td>
<td></td>
<td></td>
<td>11 µl</td>
</tr>
</tbody>
</table>

4. Mix thoroughly - Pipette up and down at least six times.
5. Spin down using countertop centrifuge.
6. Incubate overnight at 37°C. In the morning proceed to step #7.
7. Dilute M.SssI to make 1 unit M.SssI.
   M.SssI 1 µl (4 units) + RNase/DNase free water 3 µl
8. Add diluted SAM and M.SssI (Step 6) and incubate overnight at 37°C.

<table>
<thead>
<tr>
<th></th>
<th>final conc.</th>
<th>Unit</th>
<th>sample B</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAM</td>
<td>0.2</td>
<td>mM</td>
<td>1 µl (diluted)</td>
</tr>
<tr>
<td>Enzyme</td>
<td>0.09</td>
<td>unit/µl</td>
<td>2 µl (1 unit)</td>
</tr>
</tbody>
</table>

9. Stop the reaction by heating at 65°C for 20 minutes.

**Clean M.SssI-treated DNA with DNA clean & concentrator-5**

1. In a 1.5 ml microcentrifuge tube, add 2 volumes of DNA Binding Buffer to each volume of DNA sample. Mix briefly by vortexing.
2. Spin down using countertop centrifuge.
3. Transfer mixture to a provided Zymo-Spin™ Column in a Collection Tube.
4. Centrifuge at 10,000 x g for 30 seconds. Discard the flow-through.
5. Add 200 µl Wash Buffer to the column. Centrifuge at 10,000 x g for 30 seconds.
6. Repeat wash step (Step 5).
7. Transfer the column to a 1.5 ml microcentrifuge tube.
8. Add 10 µl RNase/DNase free water directly to the column matrix.
9. Centrifuge at 10,000 x g for 30 seconds to elute the DNA.
10. Measure DNA concentration using Nanodrop.
B-8. PCR Reaction for methylation measurement

doi:10.1038/nprot.2006.152

**Materials:**
- Sigma JumpStart Taq DNA polymerase Cat #. D4184
  - JumpStart Taq DNA Polymerase
  - 10x PCR Buffer without MgCl₂
- 1M MgCl₂ Cat #. AM9530G from Invitrogen
- Nuclease free water
- 10 mM dNTP mix from Promega
- Tween-20 (polyoxyethylene sorbitan monolaurate): Fisher #BP-337-100
- Gelatin: Sigma #G-9391

**PROCEDURE**

1. Taqman 10X stabilizer preparation: 0.1% Tween-20, 0.5% gelatin
   - Measure 8ml of Nuclease free water in a 15ml sterile screw capped tube.
   - Add 2ml of Tween-20 to Nuclease free water and pipet the solution repeatedly until all the Tween is mixed with Nuclease free water and to remove traces of Tween from the pipet.
   - It can be stored at room temperature until needed.
   - Weigh out 0.2g gelatin and add it to 50ml conical screw capped tube.
   - Add 20ml of Nuclease free water.
   - Heat at 37°C in an incubator to dissolve the gelatin and after it is all melted, add 0.2ml of 20% Tween-20 and bring the final volume to 40ml with nuclease free water.
   - It can be stored at room temperature until needed.

2. Oligomix preparation which contains the forward and reverse primers as well as the probe in one tube
   - Prepare the forward and reverse primers at a concentration of 300 µM and the probe at a concentration of 100 µM. Make small aliquots of the primers at these concentrations to prevent repeated freeze/thaw events.
   - Dilute the primers/probe to a working stock of 6µM (primers) and 2µM (probe). This is achieved by combining the stock solutions of the forward primer, reverse primer and probe in one tube (4 µl of the 300 µM forward primer, 4 µl of the 300 µM reverse primer and 4 µl of the 100 µM probe in 600 µl total volume):
     - Forward primer: 300 µM stock; use 4µl; 6 µM final conc.
     - Reverse primer: 300 µM stock; use 4µl; 6 µM final conc.
Probe: 100 µM stock; use 4µl; 2 µM final conc.
Nuclease free water: add 588 µl
TOTAL VOLUME: 600 µl
✓ Use 4.5 µl of this OligoMix per 30 µl MethyLight reaction. As shown in the PCR MasterMix Reaction Set-Up, this 4.5 µl volume represents the combined volumes from each of the two individual 6 µM primers and the 2 µM probe. After addition to the PCR reaction mixture, the forward/reverse primers are at a concentration of 0.3µM, and the probe at 0.1 µM.

3. PreMix Preparation:
✓ The PreMix contains all TaqMan reaction components except the primers/probe.
FOR ONE REACTION:
Taq Polymerase: 0.1 µl
25mM MgCl₂: 4.2µl
10X Buffer: 3.0µl
10X stabilizer: 3.0µl
10mM dNTPs: 0.6µl
Nuclease free water: 4.6µl
TOTAL: 15.5µl

4. Combine all agents expect bisulfite modified DNA.
✓ PreMix: 15.5 µl
OligoMix: 4.5 µl
TOTAL: 20.0 µl

5. PCR Reaction:
Load 10 µl of bisulfite modified DNA into the wells of a 96-well plate and then add 20µL of the MasterMix.
Seal caps on the plate, mix and centrifuge at 2500 rpm for 1 minute. (use Jouan centrifuge)

6. PCR program:
95°C for 10 min
Then 50 cycles of:
95°C for 15 sec
60°C for 1 min
7. MethyLight Plate Set-up:
To analyze the methylation, bisulfite-DNA is required not only for the methylation reaction, but also for the control gene reaction. Also include bisulfite converted M.SssI-treated DNA (diluted 1:10 (in duplicate)) for each methylation and control reaction.

✓ 4-point Standard Curve Set-up: For standard curve, we use bisulfite converted M.SssI-modified DNA (diluted 1:10).
   - 10 µl (in duplicate)
   - 5 µl (in duplicate)
   - 2.5 µl (in duplicate)
   - 1 µl (in duplicate)

8. MethyLight (Percentage of methylated reference (PMR) calculations):
The formula to calculate PMR values is essentially the quotient of two ratios (multiplied by 100):
\[
\left( \frac{[\text{geneX mean value for the sample}]}{[\text{control gene mean value for the sample}]} \right) / \left( \frac{[\text{geneX mean value for the M.SssI reference}]}{[\text{control gene mean value for the M.SssI reference}]} \right) \times 100
\]
The log (fluorescence) is plotted as a function of the C(t) values of each standard, and the equation of the best fit line through the points that comprise the standard curve is generated.

<table>
<thead>
<tr>
<th>ug</th>
<th>log ug</th>
<th>Ct</th>
</tr>
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<tr>
<td>10</td>
<td>1</td>
<td>25.80531</td>
</tr>
<tr>
<td>5</td>
<td>0.69897</td>
<td>26.84806</td>
</tr>
<tr>
<td>5</td>
<td>0.69897</td>
<td>26.99411</td>
</tr>
<tr>
<td>2.5</td>
<td>0.39794</td>
<td>27.75937</td>
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<td>27.43289</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>28.95447</td>
</tr>
</tbody>
</table>

Then, the C(t) value of each unknown sample is converted to a “mean value” via the standard curve best fit equation.
B-9. Cell culture of HCT116 cells

1. Preparation of complete McCoy’s 5A modified media for HCT 116 (Modified from Manasvi Shah)


Reagents

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
<th>Product #</th>
<th>size</th>
</tr>
</thead>
<tbody>
<tr>
<td>McCoy’s 5A</td>
<td>Invitrogen</td>
<td>16600-108</td>
<td>10 × 500 ml</td>
</tr>
<tr>
<td>Glutamax</td>
<td>Invitrogen</td>
<td>35050-061</td>
<td>100 ml</td>
</tr>
<tr>
<td>PBS</td>
<td>Invitrogen</td>
<td>14190-250</td>
<td>10 × 500 ml</td>
</tr>
<tr>
<td>Trypsin-EDTA</td>
<td>Invitrogen</td>
<td>25300-054</td>
<td>100 ml</td>
</tr>
<tr>
<td>FBS</td>
<td>Hyclone</td>
<td>SH30070</td>
<td>500 ml</td>
</tr>
<tr>
<td>Bottle top filter (1L)</td>
<td>Corning</td>
<td>431174</td>
<td>1 L</td>
</tr>
</tbody>
</table>

Procedure

(1) Aliquot 28 ml FBS in 50 ml falcon tube. (freeze at -20°C)
(2) Aliquot 5.5 ml glutamax in 15 ml falcon tube. (freeze at -20°C)
(3) Thaw 1 tube of glutamax aliquot and 2 tubes of FBS aliquot at 4°C overnight.
(4) Add glutamax and FBS into 500ml of McCoy’s 5A modified medium (without HEPES).
   a. 10% FBS: Add 56 ml FBS.
   b. 1% Glutamax: Add 5.5 ml Glutamax.
(5) Gently tilt the bottle to mix.
(6) Filter mixed media with bottle top filter (0.22 µm pore size) into autoclaved (121 °C for 20 min) orange cap bottle.
(7) Label as “sterile, complete” (after adding FBS and glutamax, the media is now called complete.), your initials, and the date.
(8) Store at 4°C.
2. Preparation of HCT116 cell culture

Reagents

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
<th>Product #</th>
<th>size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Culture Flask, 175 cm²</td>
<td>BD Falcon</td>
<td>353112</td>
<td>40 ea</td>
</tr>
<tr>
<td>Cell Culture Flask, 75 cm²</td>
<td>BD Falcon</td>
<td>353136</td>
<td>60 ea</td>
</tr>
</tbody>
</table>

Procedure

1. Warm complete McCoy’s 5A media to room temperature.
2. Aliquot 9 ml of complete McCoy’s 5A media in a 15ml falcon tube.
3. Take a vial of frozen cells from the liquid nitrogen tank (rack 15- box1).
4. Thaw rapidly with gentle agitation in the waterbath at 37°C
5. Slowly introduce cells to new environment by adding cells into (2).
6. Pipetting up and down several times.
7. Centrifuge at 150 x g for 5 min at room temperature. Must use centrifuge cover for HCT116 (because of BL2 lab rule: Cultured human cells are potentially biohazardous and infectious.)
8. Add 12 ml of the McCoy’s medium in T-75 flask.
10. Resuspend the cell pellet in 3 ml of medium.
11. Add resuspended cells (3 ml) into a T-75 flask with 12 ml medium.
12. Gently rotate the flask to distribute the cells evenly.
13. Label the flask (Cell type, passage #, date and initials)
14. Incubate at 37°C, 5% CO₂.
15. Check cell confluence under the microscope daily (commonly 10X) until 70-90% of cells are confluent, usually 2 days.

3. Passage of HCT116 cells

Procedure for cells in T-75 flask:

1. When cells are 70% confluent, remove all medium.
2. Wash cells with PBS, and aspirate PBS gently.
3. Add 6ml trypsin/EDTA, incubate 3 min at 37°C in CO₂ incubator.
4. Tap the flask from the side and bottom.
5. Check if cells are detached. More than 90% of cells should be floating.
6. Add 12ml of media to deactivate trypsin.
7. Pipet cells in trypsin and media into 50 ml conical tube.
8. Spin down at 150 xg, 5 min at room temperature.
10. Resuspend the cell pellet in 3 ml of medium.
11. Add resuspended cells (3 ml) into a T-75 flask with 12 ml medium.
12. Gently rotate the flask to distribute the cells evenly.
13. Label the flask (Cell type, passage #, date and initials)
14. Check cells under the microscope. (commonly 10X)
15. Incubate at 37°C, 5% CO₂.
16. Check cell confluence under the microscope daily (commonly 10X) until 70-90% of cells are confluent, usually 2 days.
4. Seed HCT116 cells in 100 mm² plate for actual experiment

Reagents
100 mm² plate, Complete media, PBS, Trypsin, 1.5 ml microcentrifuge tube, 1000p tips, 200p tips, Glass pipet

From T-75 flask
(1) When cells are 70% confluent, remove all media.
(2) Wash cells with PBS, and aspirate PBS gently.
(3) Add 6ml trypsin/EDTA, incubate 3 min at 37°C in CO₂ incubator.
(4) Tap the flask from the side and bottom.
(5) Check if cells are detached. More than 90% of cells should be floating.
(6) Add 12ml of media to deactivate trypsin.
(7) Pipet cells in trypsin and media into 50 ml conical tube.
(8) Spin down at 150 xg, 5 min at room temperature.
(9) Aspirate and discard supernatant using glass pipette with vacuum suction.
(10) Resuspend the cell pellet in 30 ml of media.
(11) Take 1 ml of media containing cells in 1.5 ml microcentrifuge tube for counting.
(12) Put 50 ml conical tube with remaining media+cells in the incubator.
(13) Apply 10 µl to each side of hemocytometer
(14) Count the # of cells in at least 4 squares on one side and 2 squares on the opposite side.

Cell counting – calculations (28 X 100 mm² plate)

30,000 cells/ml are needed, therefore add 10 ml per plate for 28 plates.

30,000 cells/ml X 10 ml = 300,000 cells for one plate

Total # of cells in all the counted squares = ____________ cells (a)

# of squares = ______ (b)

Cell density (Cell count per ml) = a/b X 10,000 = ____________cells/ml (c)

For 28 plates, prepare enough for 29 to account for any pipetting problems;

Total ml needed = 10 ml X (28+1) = 290 ml

Total cells needed = 30,000 cells/ml X 290 ml = 8,700,000 cells

\[
\text{ViCl = VfCf}
\]

\[
\text{Vi= initial volume} \\
\text{Ci=initial concentration} \\
\text{Vf= final volume} \\
\text{Cf= final concentration}
\]

\[
\text{Vi= Vf X Cf / Ci = 290 ml X 30,000 cells/ml} = ____________ ml (d)
\]

cell density (c) cell/ml

290 ml – (d) ml = __________ ml (e)

Add (e) ml of media to the 50 ml conical tube in order to suspend cells.

Dispense 10 ml into each well.
B-10. Fatty acid-BSA complex (10 mg)

Purpose: To prepare 2.5 mM fatty acid and bovine serum albumin (BSA) complex.

Materials

✓ Spatula*
✓ 100 ml beaker*
✓ 2 ml V-bottom vials (X2)*
✓ 0.2 μm Tuffryn filters and 10 ml syringes (1 for each fatty acid) (Acrodisc #PN 4192) or Millipore steriflip SCGP00525
✓ FA-free BSA (BM, #100069), MW: 68,000
✓ Sodium carbonate-Na$_2$CO$_3$ (J T Baker, #3604-01), MW: 105.99
✓ Sterile distilled water (Baxter, 2F7115)
✓ DHA [22.64 μg/μL in EtOH], MW: 328.57 (5-16-07)
✓ LA [18.6 μg/μL in EtOH], MW: 280.48 (8-3-10)

Preparation

*Glassware should be baked at 180°C for 4 hr
Day before preparation: Bake 2 sets (1 stand-by)
On prep day – ice bucket, vial screw caps (in the red lid container), and baked glassware
    1000p micropipette, tips, 1.5 ml microcentrifuge tubes, 15 ml and 50 ml conical tubes

Procedure

1. vortex stock tubes of fatty acid vigorously
2. Add 10 mg (volumes recorded below) of FA-ethanol

(SUBJECT TO CHANGE) Calculations to make up 10 mg for fatty acid stocks

DHA in EtOH – 22.64 μg/μl (dated) 5-16-07
LA in EtOH – 18.6 μg/μl (dated) 8-3-10

\[
\text{DHA} = \frac{10,000 \text{ μg}}{22.64 \text{ μg/μL}} = 441.70 \text{ μL} \\
\text{LA} = \frac{10,000 \text{ μg}}{18.6 \text{ μg/μL}} = 537.63 \text{ μL}
\]

3. Clean Nitrogen tank needles with ethanol.
4. Dry the fatty acid with N$_2$ (30 psi). Set timer for 1 hour and check every 5-10 min.
5. Aliquot 10 ml of sterile water (in 15 ml tube) and two tubes containing 20 ml of McCoy’s 5A media under the hood
6. Weigh 53 mg (0.053 g) of sodium carbonate and 3 g of fatty acid free BSA.
7. Make 0.05M Na$_2$CO$_3$

53 mg of Na$_2$CO$_3$ in 10 ml sterile H$_2$O (need 2mL per FA-BSA)
8. Make 15% BSA solution
   Add 20 ml of McCoy’s 5A media into 100mL beaker
   Gently add 3 g of BSA into the media
   **Do not stir** but let the BSA powder slowly dissolve into media

9. After drying down the fatty acid completely. Close the N₂ tank valve.
10. Add 2 ml of 0.05 M Na₂CO₃ to each FA vial.
11. Flush the vial and lid with N₂ and close the vial.
12. Vortex the vials extensively for 1 hr at RT. (every 10 min for the first 30 min
    and then every 15 min for the last 30 min to reduce the air bubbles).
13. Make 2.5 mM FA-BSA complex at the FA/BSA 3:1 mole ratio.

Sample Calculations:
DHA (MW: 328.5): 10 mg in 2 ml 0.05M Na₂CO₃;
BSA (MW: 68,000): 15% Solution
(i) Calculate the volume of 15% BSA solution needed for 10 mg DHA to obtain
FA/BSA 3/1 mole ratio.

\[
\frac{0.01 \text{ g DHA}}{328.5 \text{ (DHA mol wt)}} \times \frac{1}{3} \times \frac{1}{68000 \text{ (BSA MW)}} \times \frac{100}{15} = 4.6 \text{ (ml)}
\]

(ii) Calculate the total volume of solution needed for 10 mg DHA to make 2.5 mM
DHA-BSA complex.

\[
10 \text{ (mg of DHA)/ 328.5 (DHA mol. wt)/ 2.5 (mM)} = 0.012177 \text{ (L)} = 12.177 \text{ ml}
\]

(iii) Calculate the volume of plain McCoy’s 5A media needed for making the 2.5 mM
DHA-BSA complex.

12.177 (total volume) - 2 (FA in 0.05M Na₂CO₃) - 4.6 (15% BSA) = 5.577

\[
\begin{align*}
\text{DHA} & \quad \text{15% BSA} & \quad \text{Media} & \quad \text{2.5mM DHA-BSA} \\
2 \text{ ml} & \quad 4.6 \text{ ml} & \quad 5.577 \text{ ml} \rightarrow & \quad 12.177 \text{ ml}
\end{align*}
\]

It is difficult to completely retrieve the 2 ml FA-Na₂CO₃ from the glass vial. Therefore,
take only 95% of everything to make the DHA-BSA complex.

1.9 ml DHA + 4.37 ml 15% BSA + 5.298 ml McCoy’s 5A media.
Apply similar calculation to LA.
14. Add the following volumes of FA-Na$_2$CO$_3$ and 15% BSA to a 50 ml conical tube and let it mix for 5 min. Then add the volume of media to a 50 ml conical tube for individual FA.

<table>
<thead>
<tr>
<th></th>
<th><strong>DHA TUBE</strong></th>
<th></th>
<th><strong>LA TUBE</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>15% BSA (ml)</td>
<td>4.37 ml</td>
<td>10 mg</td>
<td>5.12 ml</td>
</tr>
<tr>
<td>McCoy’s 5A (ml)</td>
<td>5.3 ml</td>
<td></td>
<td>6.53 ml</td>
</tr>
<tr>
<td>Fatty acid – Na$_2$Co$_3$</td>
<td>1.9 ml</td>
<td></td>
<td>1.9 ml</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>11.57 ml</strong></td>
<td></td>
<td><strong>13.55 ml</strong></td>
</tr>
</tbody>
</table>

15. Flush the tubes and lids with N$_2$.

16. Shake the tubes on belly dancer for 1/2 hr at RT.

17. Turn the UV light on before starting to do the filter sterilization, filter-sterilize the FA-BSA complex using Millipore steriflip under sterile hood.

18. Aliquot 500 µl of each into 1.7 ml microcentrifuge tubes and label the box. Store at -20°C.

19. Aliquots have to be used within **60 days**.

The final concentration of FA is 2.5 mM. The solution is 5.67% BSA. Working concentration of FA is 50 µM. This solution is 0.11% BSA.
**B-11. 5-Aza-2-deoxycytidine (AZA) dilution (MW=228.2)**

Methyltransferase inhibitor  
Cat #. sc-202424 from Santa Cruz Biotechnology

**100 mM (100,000 µM) Stock**
-Dissolve 25mg of AZA in 1.095 ml of PBS under the hood. It gives us 100mM solution.  
-Aliquot in sterile microcentrifuge tubes, 50 µl per tube under the hood. We have 21 aliquots.  
-Store at -80°C.

**Dilution**
- **Step 1 (1 ml of 1000 µM)**  
  1:100 dilution (1000 µM) by taking 10 µl 100mM stock + 990 µl media

- **Step 2 (50 ml of 1µM media)**  
  1:1000 dilution (1µM) by taking 50 µl Step #1 solution + 49,950 µl media

- **Step 3 (50 ml of 2µM media)**  
  1:500 dilution (2 µM) by taking 100 µl of Step #1 solution + 49,900 µl media
B-12. DNA extraction and quantification from cultured cells

QIAamp DNA mini kit Cat #: 51304 from Qiagen
RNase A Cat #: 19101 from Qiagen
Cell scraper Cat #: 179707 from Thermo Scientific.

Use the appropriate number of cells (do not use more than 5 x 10^6 cells)

1. Remove media
2. Wash 3 times with PBS (make sure you add PBS to the plate wall)
3. Add 600 µl of PBS and scrape the cells from plate using cell scraper.
4. Put the solution into an microcentrifuge tube (1.5ml)
5. Place tubes on ice (Store at -80°C until DNA extraction)
6. Thaw cells in ice and centrifuge for 5 min at 300 x g at room temperature.
   Remove the supernatant completely and discard, taking care not to disturb the cell pellet.
7. Resuspend cell pellet in PBS to a final volume of 200 µl.
8. Add 20 µl protease K (in kit).
9. Add 4 µl RNase A (100 mg/ml), vortex, spin down using countertop centrifuge,
   and incubate at room temperature for 2 min.
10. Add 200 µl Buffer AL and vortex for 15 s.
11. Incubate at 56°C for 10 min using Eppendorf thermomixer and spin down using
    countertop centrifuge.
12. Add 200 µl of 100% ethanol to the sample, and vortex for 15 s and spin down
    using countertop centrifuge.
13. Carefully apply 700 µl of solution from step 13 to the QIAamp Mini spin
    column/collection tube setup and close the cap.
14. Centrifuge at 6000 x g for 1 min. Place the QIAamp Mini spin column in a clean
    2 ml collection tube or discard the solution which passed through the filter into
    the collection tube in order to reuse it.
15. Repeat 13 and 14 until filter all of the solution generated in step 13.
16. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1.
17. Close the cap and centrifuge at 6000 x g for 1 min.
18. Place the QIAamp Mini spin column in an empty or new 2 ml collection tube.
19. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2.
20. Close the cap and centrifuge at full speed (20,000 x g) for 3 min.
21. Place the QIAamp Mini spin column in a new (not emptied) 2 ml collection tube
    and centrifuge at full speed for 1 min.
22. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube and
    add 100 µl Buffer AE.
23. Incubate at room temperature for 5 min, and then centrifuge at 6000 x g for 1 min.
24. Repeat #22 for a second elution step with an additional 100 µl Buffer AE in same
    tube.
25. Measure DNA concentration using Nanodrop. (Make sure to select DNA option).
   - Recommendation A260:A230 >1.7,  A260:A280 >1.8
B-13. ELISA Global DNA methylation quantification

Purpose: To determine the levels of global DNA methylation

Materials:
- MethylFlash Methylated DNA Quantification kit (Colorimetric) Cat #. P-1034 from Epigentek
- Input DNA: 100 ng per reaction (dilute each sample to 20 ng/µl DNA and take 5 µl of each diluted sample to have 100 ng per reaction)
- Distilled water, Cat #. 15230 from Gibco
- Add 13 ml of ME1 (10X washing butter) from kit to 117 ml of distilled water (pH 7.2-7.5).
- Multichannel pipet and tips
- Foil
- Turn on the microplate reader (Synergy HT, Bio-Tek), prepare protocol “450nm”
- Turn on the incubator 37°C

Procedure
1. Single point control preparation; dilute ME4 (positive control from kit) to 5 ng/µl (1 µl of ME4 + 3 µl of TE buffer).

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
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<tbody>
<tr>
<td>A</td>
<td>ME3</td>
<td>ME4</td>
<td>D2</td>
<td>LA3</td>
<td>DB4</td>
<td>LB5</td>
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<td>B</td>
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<td>ME4</td>
<td>LA2</td>
<td>DB3</td>
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<tr>
<td>C</td>
<td>ME3</td>
<td>ME4</td>
<td>DB2</td>
<td>LB3</td>
<td>Con5</td>
<td>Aza6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Con1</td>
<td>DB1</td>
<td>LB2</td>
<td>Con4</td>
<td>Aza5</td>
<td>But6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>Aza1</td>
<td>LB1</td>
<td>Con3</td>
<td>Aza4</td>
<td>But5</td>
<td>D6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>But1</td>
<td>Con2</td>
<td>Aza3</td>
<td>But4</td>
<td>D5</td>
<td>LA6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>D1</td>
<td>Aza2</td>
<td>But3</td>
<td>D4</td>
<td>LA5</td>
<td>DB6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>LA1</td>
<td>But2</td>
<td>D3</td>
<td>LA4</td>
<td>DB5</td>
<td>LB6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. Add 80 µl of ME2 (binding solution) to each well.
3. Add 1 µl of ME3 (negative control), 1 µl of Diluted ME4 (positive control, prepared in Step #1), and 100 ng of DNA into designated wells.
4. Mix gently. Ensure the solution coats the bottom of the well evenly.
5. Cover strip plate with plate seal or parafilm.
6. Incubate at 37 °C for 90 min.
7. Wash each well with 150 µl of the Diluted ME1 for 3 times. Discard in the sink and slightly tap on a paper towel.
8. Dilute ME5 (capture antibody) with the Diluted ME1 (at 1:1000 ratio)
   - 3 µl ME5
   - 3000 µl diluted ME1
9. Add 50 μl of the diluted ME5 to each well.
10. Cover and incubate at room temperature for 60 min.
11. Remove the diluted ME5 solution from each well. Discard in the sink and slightly tap on a paper towel.
12. Wash each well with 150 μl of the diluted ME1 for 3 times.
13. Dilute ME6 (detection antibody) (at 1:2000 ratio) with the Diluted ME1.
   ▪ 3 μl ME6
   ▪ 6,000 μl diluted ME1
14. Add 50 μl of the diluted ME6 to each well.
15. Cover and incubate at room temperature for 30 min.
16. Remove the diluted ME6 solution from each well. Discard in the sink and slightly tap on a paper towel.
17. Wash each well with 150 μl of the diluted ME1 for 4 times.
18. Dilute ME7 (enhancer antibody) (at 1:5000 ratio) with the Diluted ME1.
   ▪ 1 μl ME7
   ▪ 4,999 μl diluted ME1
19. Add 50 μl of the diluted ME7 to each well.
20. Cover and incubate at room temperature for 30 min.
21. Remove the diluted ME7 solution from each well. Discard in the sink and slightly tap on a paper towel.
22. Wash each well with 150 μl of the diluted ME1 for 5 times.
23. Add 100 μl of ME8 (developer solution) to each well.
24. Incubate at room temperature for 1-10 min away from light UNTIL the positive control turns medium blue (around 2 minutes)
25. Add 50 μl of ME9 (stop solution).
26. Measure absorbance every 2 minutes for 10 measurements using the kinetic analysis protocol at 450 nm on the microplate reader.
B-14. ELISA Apoptosis Assay

Purpose: To determine apoptotic cells in cell culture experiment

Materials:
- ELISA kit: Cell death detection ELISA Plus from Roche- (BM# 11774425)
- Reconstitute working solutions (refer to manual, pg 5)
- Bottle 1: anti-histone-biotin: each bottle, reconstitute in 450 µL double distilled H₂O for 10 min and mix well (store at 2-8°C up to 2 months)
- Bottle 2: anti-DNA-POD- reconstitute with 450 µL double distilled H₂O for 10 min and mix well (store at 2-8°C upto 2 months)
- Bottle 3: Positive control - reconstitute with 450 µL double distilled H₂O for 10 min and mix well (store at 2-8°C up to 2 months)
- Bottle 7: ABTS Tablets- dissolve 1 tablet in 5 ml of substrate buffer (vial 6). (100 µL / sample). This is light sensitive and hence store container in a foil wrap (store for 1 month protected from light)
- Complete media (without γ-INF): Make several aliquots to avoid contamination of stock solution
- PBS: Make several aliquots to avoid contamination of stock solution
- Trypsin: Make several aliquots to avoid contamination of stock solution
- Label 2 sets of 2 ml and 1 set of 0.67 ml microcentrifuge tubes: floaters collection (2 ml tubes for step 1), counting cells (2 ml tubes for counting) supernatant collection (0.67 ml tubes for step 13).
- Fill coulter counter cups the day before the assay with 10 ml.

Procedure
1. Under sterile conditions in the hood: swirl the 6-well plates and collect the media and floating cells into the 1st set of 2 ml microcentrifuge tubes. If more than 1.5 ml of media was in each well, prepare more microcentrifuge tubes to collect media and floating cells.
2. Add 1 ml of sterile PBS into the wells and put them in the 37°C incubator for counting later. If you have a plan to count cells later, add 1 ml of complete media.
3. Centrifuge the first set of microcentrifuge tubes at 500 x g for 5 min at RT.
4. Remove supernatant using 1ml pipette manually without disturbing the pellet (pellet cannot be seen most of the time so leave a little media on this step).
5. Resuspend pellet in 1 ml PBS.
6. Finger flick the tubes to mix.
7. Centrifuge again at 500 x g for 5 min at RT to wash and remove the excess media.
8. Remove as much of the supernatant as possible (without disturbing the pellet) and discard it. Use a p-200 pipette.
9. Resuspend cell pellet with 100 µL lysis buffer (bottle 5 of Cell death detection ELISA kit)
10. Mix thoroughly and **incubate** sample for **30 min** at **RT**

11. During incubation, prepare the immunoreagent (manual: pg 6)

   **For 10 tests**: 40 µL of reconstituted reagent (bottle 1) + 40 µL of reconstituted reagent (bottle 2) + 720 µL of incubation buffer from bottle 4. Mix well.

12. After incubation, centrifuge lysate at 500 x g for 10 min, at **RT**. (*Pellets out the intact cells and nuclei etc leaving the cytosolic fraction in the supernatant for the assay*)

13. Transfer the supernatant carefully into another 0.67 ml microcentrifuge tube without shaking the pellet.


   Use 20µL of
   - Well (A-1) – Background (Bottle 4 of kit)
   - Well (B-1) – Lysis Buffer (Bottle 5 of kit)
   - Well (C-1) – Positive control (Bottle 3 of kit)

15. Transfer **20 µL** of all samples into microtiter plate (MP). Pipette into the **middle** of the MP well.

16. Add to each well **80 µL** of the immunoreagent using multiple channel pipet.

17. Remove all bubbles from the wells in the MP with a pipette tip (use different tip for each well).

18. Cover the MP with an adhesive cover and foil. Incubate on a MP shaker under gently shaking (300 rpm) for **2hr** at 15-25°C.

19. Empty the solution from the MP thoroughly by **tapping** (tape both sides of the module strips in plateholder to secure them).

20. Rinse each well **3x with 250 µL** of incubation buffer (bottle 4).

21. Empty the solution carefully by tapping.

22. In the dark (lights off), pipette to each well **100 µL** ABTS solution (light sensitive).

23. Measure every 2 minutes for 10 measurements using the kinetic analysis protocol at **405nm** against ABTS solution as a blank (reference wavelength approx 490nm, also test at 450nm).

**COUNTING**

1. Remove the plates from the incubator

2. Swirl the plates with the PBS and aspirate the PBS from the plates

3. Add 0.5 ml Trypsin and put them in the incubator for 3 minutes @ 37°C.

4. Add 1ml media into each of the plates with the trypsin. Mix by pipetting up and down.

5. Add 20 µl of cell suspension to 10 ml of coulter counter solution.

6. Count the cells using coulter counter.
   - Repeat 3 times or until consistent cell numbers are obtained.

7. Calculate the cell density and total number of apoptotic cells.

8. Use this count to calculate the apoptotic index.

9. Apoptotic Index = Optical Density/ # adherent cells
B-15. Gene specific methylation using PCR protocol


Purpose: To determine gene-specific promoter methylation

Material:
- Methyl-Profiler DNA methylation PCR array Cat #. MeAH-121 from SABiosciences
- Methyl-Profiler Enzyme Kit Cat #. MeA-03 from SABiosciences
- RT² SYBR Green qPCR mastermix Cat #. 330520 from SABiosciences

This protocol is for a 24-gene, 96-well PCR Array and one DNA sample.

Restriction digestion using enzyme kit

1. Calculate sample volume to have 1 μg of DNA.
2. Prepare a reaction cocktail as indicated below. Use 1 μg DNA.

<table>
<thead>
<tr>
<th>RNase/DNase-free H₂O</th>
<th>___ μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X Digestion Buffer</td>
<td>26 μl</td>
</tr>
<tr>
<td>Genomic DNA (1 μg)</td>
<td>___ μl</td>
</tr>
<tr>
<td>Final cocktail volume</td>
<td>120 μl</td>
</tr>
</tbody>
</table>

3. Vortex and spin down using countertop centrifuge.
4. Label 4 1.5 ml microcentrifuge tubes for one sample. (Mo, Ms, Md and Msd)
5. Add 28 μl of a reaction cocktail from Step #2 to each labeled microcentrifuge tube. All four tubes must contain equal amounts of DNA.
6. Add RNase/DNase free water and enzymes as indicated below. (DO NOT vortex enzymes) Pipette up and down to thoroughly, yet gently, mix the components. Spin down using countertop centrifuge.

* Mock Digest (Mo) No enzymes are added in this reaction. The product of the mock digestion represents the total amount of input DNA for real-time PCR detection.

* Methylation Sensitive Digest (Ms) Cleavage with a methylation-sensitive enzyme will digest unmethylated and partially methylated DNA. The remaining (hyper)-methylated DNA will be detected by real-time PCR.

* Methylation Dependent Digest (Md) Cleavage with a methylation-dependent enzyme will preferentially digest methylated DNA. The remaining unmethylated DNA will be detected by real-time PCR.
* **Double Digest (Msd)** Both enzymes are added in the double digest, and all DNA molecules (both methylated and unmethylated) will be digested. This reaction measures the background and the fraction of input DNA refractory to enzyme digestion.

<table>
<thead>
<tr>
<th></th>
<th>Mo</th>
<th>Ms</th>
<th>Md</th>
<th>Msd</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RNase/DNase-free H₂O</strong></td>
<td>2 μl</td>
<td>1 μl</td>
<td>1 μl</td>
<td></td>
</tr>
<tr>
<td><strong>Cocktail from previous step</strong></td>
<td>28 μl</td>
<td>28 μl</td>
<td>28 μl</td>
<td>28 μl</td>
</tr>
<tr>
<td><strong>Enzyme A</strong></td>
<td></td>
<td>1 μl</td>
<td></td>
<td>1 μl</td>
</tr>
<tr>
<td><strong>Enzyme B</strong></td>
<td></td>
<td></td>
<td>1 μl</td>
<td>1 μl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>30 μl</td>
<td>30 μl</td>
<td>30 μl</td>
<td>30 μl</td>
</tr>
</tbody>
</table>

7. Incubate all four tubes at 37°C overnight.
8. Stop the reactions by heat-inactivating the enzymes at 65°C for 20 minutes.
9. Store at -20°C before using samples.

**Setting up the PCR Reactions**

1. Thaw 4 enzyme reaction tubes (for one sample) from the previous step.
2. In each tube, add 300 μl RNase/DNase free water and 330 μl PCR master mix following the table below.
3. Vortex and spin down using countertop centrifuge.

<table>
<thead>
<tr>
<th></th>
<th>Mo</th>
<th>Ms</th>
<th>Md</th>
<th>Msd</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RNase/DNase-free H₂O</strong></td>
<td>300 μl</td>
<td>300 μl</td>
<td>300 μl</td>
<td>300 μl</td>
</tr>
<tr>
<td><strong>PCR Master Mix</strong></td>
<td>330 μl</td>
<td>330 μl</td>
<td>330 μl</td>
<td>330 μl</td>
</tr>
<tr>
<td><strong>Mo digest</strong></td>
<td>30 μl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ms digest</strong></td>
<td></td>
<td>30 μl</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Md digest</strong></td>
<td></td>
<td></td>
<td>30 μl</td>
<td></td>
</tr>
<tr>
<td><strong>Msd digest</strong></td>
<td></td>
<td></td>
<td></td>
<td>30 μl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>660 μl</td>
<td>660 μl</td>
<td>660 μl</td>
<td>660 μl</td>
</tr>
</tbody>
</table>

4. Carefully add 25 μl of the Mo cocktail (described in table above) to each well in rows A & B of the 96-well PCR Array, 25 μl of the Ms cocktail to each well in rows C & D, 25 μl of the Md cocktail to each well in rows E & F, and 25 μl of the Msd cocktail to each well in rows G & H as indicated below. (This is for one sample.)
5. After loading the plate, carefully seal the plate or cap the wells. Centrifuge the plates briefly to remove air bubbles at 2000 rpm for 1 minute.

Running the PCR Reactions using ABI 7900HT

To run the reactions, use the four segment cycling program shown below.

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95°C</td>
<td>10 minutes</td>
</tr>
<tr>
<td>3</td>
<td>99°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>40</td>
<td>97°C</td>
<td>15 seconds</td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>1 minute²</td>
</tr>
</tbody>
</table>

**Melting curve segment**: According to instrument recommendations

**Data analysis**

1. Obtaining the raw Threshold Cycle (Ct) Values
   - Note that you must compare multiple plates so be sure that the settings for all plates are identical (Same baseline and threshold value)
   - **Baseline**: Using the Linear View of the amplification plots, set the instrument to use the readings from cycle number 2 through the cycle just before the earliest visible amplification, usually between cycle 10 and 15.
   - **Threshold Value**: Using the Log View of the amplification plots, place the threshold above the background signal but within the lower third of the linear portion of the amplification curves.

2. Exporting Ct Values
3. Excel-Based Data Analysis Template: Download the Methyl-Profiler PCR Array Excel-based data analysis template that matches the gene panel and plate format that you used from the SABiosciences website at: http://www.sabiosciences.com/methylationdataanalysis.php

4. Paste in the Ct value data and analyze the automatically generated results by following the directions in the “Instructions” worksheet of the Excel file.

The ΔCt Data Analysis Method

The fraction of DNA in each digest is calculated by normalizing the DNA amount to the amount of digestible DNA. The amount of digestible DNA is equal to the total amount of DNA (determined from the mock digest) minus the amount of DNA resistant to DNA digestion (determined from the double digest).

Hypermethylated (HM) DNA Fraction:

$$F_{HM} = \frac{C_{Ms}}{C_{Mo} - C_{Msd}} = \frac{2^{-\Delta Ct(M_s)}}{2^{-\Delta Ct(M_o)} - 2^{-\Delta Ct(M_{sd})}}$$

Unmethylated (UM) DNA Fraction:

$$F_{UM} = \frac{C_{Md}}{C_{Mo} - C_{Msd}} = \frac{2^{-\Delta Ct(M_d)}}{2^{-\Delta Ct(M_o)} - 2^{-\Delta Ct(M_{sd})}}$$

Intermediately Methylated (IM) DNA Fraction:

$$F_{IM} = 1 - F_{HM} - F_{UM}$$

DNA Copies Resistant (R) to Enzyme Digestion:

$$F_R = \frac{C_{Msd}}{C_{Mo}} = \frac{2^{-\Delta Ct(M_{sd})}}{2^{-\Delta Ct(M_o)}} = 2^{\Delta Ct(M_{sd}) - \Delta Ct(M_o)} = 2^{-\Delta Ct(M_{sd}) \cdot M_o}$$

For Example:

<table>
<thead>
<tr>
<th>Symbol</th>
<th>M_o</th>
<th>M_s</th>
<th>M_d</th>
<th>M_{sd}</th>
<th>R</th>
<th>HM</th>
<th>UM</th>
<th>IM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCNA1</td>
<td>23.16</td>
<td>27.11</td>
<td>24.89</td>
<td>36.51</td>
<td>0.0995 %</td>
<td>6.47 %</td>
<td>30.15 %</td>
<td>63.38 %</td>
</tr>
</tbody>
</table>

$$F_{HM} = 2^\Delta Ct(M_s) / (2^\Delta Ct(M_o) - 2^\Delta Ct(M_{sd})) = 0.0647 \text{ or } 6.47 \%$$

$$F_{UM} = 2^\Delta Ct(M_d) / (2^\Delta Ct(M_o) - 2^\Delta Ct(M_{sd})) = 0.3015 \text{ or } 30.15 \%$$

$$F_{IM} = 1 - F_{HM} - F_{UM} = 1 - 0.0647 - 0.3015 = 0.6338 \text{ or } 63.38 \%$$

$$F_{R} = 2^\Delta Ct(M_{sd} - M_o) = 2^\Delta Ct(36.51 - 23.16) = 0.0095 \%$$
B-16. Histone H3 acetylation detection from HCT 116 cells

Purpose: To determine the levels of histone H3 acetylation

Materials:
- EpiQuik Total Histone H3 acetylation detection Kit (Colorimetric) (P-4030, Epigentek)
- Distilled water, Cat. # 15230 from Gibco
- Multichannel pipet and tips
- Turn on the microplate reader.
- Histone proteins were extracted as per Kit (OP-0006, Epigentek) instructions and the sample concentrations were measured using a BCA Protein Assay Kit (23225, Pierce)

Procedure

1. Prepare standard control dilutions.

<table>
<thead>
<tr>
<th></th>
<th>dH₂O</th>
<th>Stock (100 ng/µl)</th>
<th>Final amount</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>stock</td>
<td>0 µl</td>
<td>2 µl of stock</td>
<td></td>
<td>100 ng/µl</td>
</tr>
<tr>
<td>A_50</td>
<td>5 µl</td>
<td>5 µl of stock</td>
<td>5</td>
<td>50 ng/µl</td>
</tr>
<tr>
<td>B_25</td>
<td>5 µl</td>
<td>5 µl of vial A</td>
<td>5</td>
<td>25 ng/µl</td>
</tr>
<tr>
<td>C_12</td>
<td>5.42 µl</td>
<td>5 µl of vial B</td>
<td>5.42</td>
<td>12 ng/µl</td>
</tr>
<tr>
<td>D_6</td>
<td>5 µl</td>
<td>5 µl of vial C</td>
<td>5</td>
<td>6 ng/µl</td>
</tr>
<tr>
<td>E_3</td>
<td>5 µl</td>
<td>5 µl of vial D</td>
<td>5</td>
<td>3 ng/µl</td>
</tr>
<tr>
<td>F_1.5</td>
<td>5 µl</td>
<td>5 µl of vial E</td>
<td>10</td>
<td>1.5 ng/µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>2 µl</td>
<td>0</td>
<td></td>
<td>0 ng/µl</td>
</tr>
</tbody>
</table>

2. Dilute C1 (wash buffer) with distilled water at a 1:9 ratio. (e.g. 1 ml of C1 + 9 ml of distilled water)

3. Add 50 µl of C2 (antibody buffer) into each well.

4. Add 100 ng of sample histones. Add 1 µl of standard controls.
5. Mix and cover the plate with parafilm.
6. Incubate at room temperature for 2 hr.
7. Wash wells with 150 µl of diluted C1 from kit three times.
8. Dilute C3 (detection antibody) with C2 (antibody buffer). (1 µl of C3 + 1000 µl of C2).
10. Incubate at room temperature for 1 hr on an orbital shaker (100 rpm).
11. Wash wells with 150 µl of diluted C1 six times.
12. Add 100 µl of C4 (color developer) and incubate at room temperature 2-10 minutes while protected from light.
13. When the color of standard control turns medium blue (around 2 minutes, keep watching), add 50 µl of C5 (stop solution).
14. Measure absorbance every 2 minutes for 10 measurements using the kinetic analysis protocol at 450nm on the microplate reader.

\[
\text{Amount (ng/mg protein)} = \frac{\text{OD (sample} - \text{blank)}}{\text{Protein (µg)} \times \text{slope}} \times 100
\]
B-17. Histone H4 acetylation detection from HCT 116 cells

Purpose: To determine the levels of histone H4 acetylation

Materials:
- EpiQuik Total Histone H4 acetylation detection Kit (Colorimetric) (P-4032, Epigentek)
- Distilled water, Cat. # 15230 from Gibco
- Multichannel pipet and tips
- Turn on the microplate reader.
- Histone proteins were extracted as per Kit (OP-0006, Epigentek) instructions and the sample concentrations were measured using a BCA Protein Assay Kit (23225, Pierce)

Procedure

1. Prepare standard control dilutions.

<table>
<thead>
<tr>
<th>stock</th>
<th>dH2O</th>
<th>Stock (100 ng/µl)</th>
<th>Final amount</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>stock</td>
<td>0 µl</td>
<td>2 µl of stock</td>
<td>100 ng/µl</td>
<td></td>
</tr>
<tr>
<td>A_50</td>
<td>5 µl</td>
<td>5 µl of stock</td>
<td>5</td>
<td>50 ng/µl</td>
</tr>
<tr>
<td>B_25</td>
<td>5 µl</td>
<td>5 µl of vial A</td>
<td>5</td>
<td>25 ng/µl</td>
</tr>
<tr>
<td>C_12</td>
<td>5.42 µl</td>
<td>5 µl of vial B</td>
<td>5.42</td>
<td>12 ng/µl</td>
</tr>
<tr>
<td>D_6</td>
<td>5 µl</td>
<td>5 µl of vial C</td>
<td>5</td>
<td>6 ng/µl</td>
</tr>
<tr>
<td>E_3</td>
<td>5 µl</td>
<td>5 µl of vial D</td>
<td>5</td>
<td>3 ng/µl</td>
</tr>
<tr>
<td>F_1.5</td>
<td>5 µl</td>
<td>5 µl of vial E</td>
<td>10</td>
<td>1.5 ng/µl</td>
</tr>
<tr>
<td>water</td>
<td>2 µl</td>
<td></td>
<td>0</td>
<td>0 ng/µl</td>
</tr>
</tbody>
</table>

2. Dilute C1 (wash buffer) with distilled water at a 1:9 ratio. (e.g. 1 ml of C1 + 9 ml of water)
3. Add 50 µl of C2 (antibody buffer) into each well.
4. Add 2 µg of sample histones. Add 1 µl of standard control.
5. Mix and cover the plate with parafilm.
6. Incubate at room temperature for 1-2 hr.
7. Prepare the Detection solution.
   - 3 µl of C3 (detection antibody) + 1.5 µl of signal report solution + 30 µl of diluted C1
   - Mix and incubate at room temperature for 10 min.
   - Add 60 µl of signal enhancer.
   - Mix and incubate at room temperature for 15 min.
   - Add 2910 µl of diluted C1.
8. Wash wells with 150 µl of diluted C1 three times.
9. Add 50 µl of detection solution.
10. Incubate at room temperature for 1 hr on an orbital shaker (100 rpm).
11. Wash wells with 150 µl of diluted C1 six times.
12. Add 100 µl of C4 (color developer) and incubate at room temperature 2-10 minutes away protected from light.
13. When the color of standard control turns medium blue (around 2 minutes, keep watching), add 50 µl of C5 (stop solution).
14. Measure absorbance every 2 minutes for 10 measurements using the kinetic analysis protocol at 450nm on the microplate reader.

\[
\text{Amount (ng/mg protein)} = \frac{\text{OD (sample – blank)}}{\text{Protein (µg)} \times \text{slope}} \times 100
\]
VITA

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Thesis Title: Effects of soy isoflavone on bone metabolism of ovariectomized rats in vivo and ex vivo

Bachelor of Science, Food & Nutrition (Feb. 2003)
Department of Food & Nutrition, Hanyang University, Republic of Korea

Honors and Awards


Graduated third in class in Dept. of Food & Nutrition, Hanyang University 2003

Awarded second place in the 12th Annual Student Research Week Oral competition, Medicine/Human Nutrition/Biomedical Engineering, Texas A&M University 2009

Awarded second place in the ASN Diet and Cancer RIS poster competition, Experimental Biology 2009, New Orleans, LA

Recipient of Sigma-Xi Grant-in-aid of research, 2010

Publications


Cho Y, Turner ND, Davidson LA, Chapkin RS, Carroll RJ, Lupton JR. Nutriepigenetic regulation of apoptosis-related genes by DHA and butyrate. (Submitted)

Cho Y, Turner ND, Davidson LA, Chapkin RS, Carroll RJ, Lupton JR. A chemoprotective fish oil/pectin diet enhances apoptosis via Bcl-2 promoter methylation in carcinomas from the rat AOM-induced colon cancer model. (Submitted)