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Mridvika

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Maurice R. Bornstein
Major professor

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DIETARY MODULATION OF TUMORIGENESIS AND GENE EXPRESSION IN THE MIN MOUSE, A GENETIC MODEL FOR HUMAN COLORECTAL CANCER

BY

Mridvika

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ABSTRACT

DIETARY MODULATION ON TUMORIGENESIS AND GENE EXPRESSION IN THE MIN MOUSE, A GENETIC MODEL FOR HUMAN COLORECTAL CANCER

By

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Mutations in the adenomatous polyposis coli (*Apc*) gene have been identified in human colorectal cancer and in familial adenomatous polyposis. The Apc^{Min} (or Min) mouse carries a germline mutation in the Apc gene and develops adenomas in the small intestine and colon, making it an ideal model for the study of intestinal cancers.
The purpose of this dissertation was two-fold: 1) to examine the effects of three dietary treatments (food restriction, feeding a defatted flax/whole-wheat-flour based diet, and feeding a black bean-based diet) on tumor multiplicity and growth in small intestine and colon in the Min mouse; 2) to provide, if possible, a molecular basis for the effects observed. The non-steroidal anti-inflammatory drug (NSAID) sulindac was used as the positive control.

The first study demonstrated that dietary treatments could alter intestinal tumorigenesis. Tumor number in the small intestine decreased ($P<0.05$) from 76 ± 5.8 in the casein fed control group to 45 ± 5.3 in the sulindac group (42% decrease), 53 ± 5.5 in the flax/wheat group (30% decrease), 55 ± 5.7 in the black bean group (28% decrease) and 48 ± 9.9 in the diet restricted group (36% decrease). Sulindac reduced average small intestine tumor diameter from 1.3 to 1.0 mm ($p<0.01$) while black bean and flax/wheat diets increased tumor diameter ($p<0.05$). Colon tumor numbers did not differ

significantly among groups, but there was an increase in colon tumor size in mice fed black bean and flax/wheat diets compared to the control group.

Since energy restriction in study one had the most dramatic impact on tumor growth, the second study focused on changes in tumor number and size in Min mice after 3, 6 and 10 weeks of 30% energy restriction. Complimentary DNA arrays were used to determine changes in expression of 1,200 mouse genes in small intestinal tumor tissue from mice fed ad libitum or restricted in energy intake. The results indicate a significant inhibition of tumor multiplicity and size in the small intestine after 3, 6 and 10 weeks of energy restriction ($p<0.05$). In the colon, the changes in adenoma number and volume caused by energy restriction were not significant. Of 1,200 genes studies, eight were overexpressed (≥ 1.6 fold) and 13 were underexpressed (≥ 1.6 fold) in restricted mice in comparison to control mice. In tumors from the energy restricted mice 30% percent of the underexpressed genes were oncogenes and 50% of the overexpressed genes were immune function proteins.

This research clearly demonstrated that energy restriction will inhibit small intestine tumor growth in Min mice. Furthermore, tumors in mice fed fewer calories had reduced expression of oncogenes and increased expression of genes related to immune function.

This dissertation is lovingly dedicated to my family, Shakun and Sarban Brar, Anu and Murty Vyakarnam, Enric and Rosa Maria, and my husband, Josep

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LITERATURE REVIEW

LITERATURE REVIEW

This year nearly 552,200 Americans are expected to die of cancer—an average of more than 1,500 people a day. Cancer is the second leading cause of death in the United States, exceeded only by heart disease. In the United States, one of every four deaths is from cancer. Colorectal cancers are the third most common cancers in men and women with an estimated 93,800 new cases of colon cancer and 36,400 of rectal cancer in the year 2000. The American Cancer Society has estimated 56,300 deaths (47,700 from colon cancer, 8,600 from rectal cancer) for the year 2000, accounting for about 11% of all cancer deaths. On a positive note, incidence rates for colon and rectal cancers have declined significantly from 1992 to1996 (-2.1% per year). Research suggests that this improvement may be due to early detection due to increased screening and polyp removal, preventing progression of polyps to invasive cancers (ACS, 2000).

Diet and Colon Cancer

Colon cancer is one of the most common cancers in the Western world. Environmental factors are the predominant causal factors as exemplified by a change in the incidence of colon cancer when people emigrate from a low- to a high-incidence country (Haenszel and Kurihara, 1968). It has been suggested that a diet high in energy, fat (Stemmermann *et al.*, 1984), and meat content (Enstrom, 1975) and low in fiber content (Burkitt, 1969; Giovannucci *et al*, 1994), (i.e. a Western style diet) may be responsible (Kotake *et al.*, 1995). Conversely a diet high in fruits and vegetables is linked to a lower risk for developing colon cancer (Hertog *et al.*, 1996). Other dietary components reported to

influence colon cancer risk include micronutrients such as vitamins C and D, calcium (Holt, 1999; Slattery et al, 1988), and selenium to name a few.

The non-nutrient components of food and their anti-cancer properties have only been studied extensively in the past decade or two. Phytochemicals, the naturally occurring plant constituents, have been linked to reduced cancer risk. Phenolic phytochemicals are the largest category of phytochemicals and are most widely distributed in the plant kingdom. Food sources of these compounds range from grains such as whole wheat, soy and dry beans to vegetables such as onion, kale, and celery and fruits such as raspberries, apples, cherries and cranberries (King and Young, 1999). Phenolic compounds in tea and coffee have also been shown to inhibit mutagenesis (Stich *et al.*, 1982). The anti-carcinogenic properties of these compounds might be linked to their anti-oxidant and free-radical scavenging properties.

Genetic Basis of Colon Cancer

Cancers differ from other genetic diseases, as they require not one but several mutations (Vogelstein and Kinzler, 1993). The "multiple hit" hypothesis for the development of cancer is supported by the relationship between the incidence of most human cancers and age suggesting that multiple mutations occurring over decades lead to development of cancer (Knudson, 1985). Colon cancer is understood to be a result of several germline and somatic genetic mutations. Because the development of colon tumors occurs through well-defined morphological stages, it has been possible to establish an approximate order in which these mutations occur (Fearon and Vogelstein, 1990). Tumor suppressor genes are negative regulators of cell growth and their loss by mutation results in loss of the

crucial "brake" on tumor growth. The initiating mutation in colon cancer is in the tumor suppressor gene, adenomatous polyposis coli (*Apc*). The second hit can either be the loss of the other allele of *Apc*, or the mutation of one of the other genes implicated in colon cancer. Mutations in the *Apc* gene lead to the formation of benign adenomas. Mutation of the *ras* gene in one of the benign adenomas can lead to further clonal expansion. This can be followed by additional mutations in genes such as the p53 or the *DCC* (Deleted in Colon Cancer) gene that can lead to the progression of the benign adenoma to the malignant carcinoma stage (Fearon and Vogelstein, 1990). A better understanding of genetic alterations that are involved in the onset and progression of colon cancer has provided a keener focus on the gene-nutrient interplay in colon cancer and its role in incidence and progression of the disease.

Colon Cancer and the *Apc* Gene

Mutation of the *Apc* gene is thought to be one of the earliest mutations according to the multi-step genetic model for colorectal tumorigenesis proposed by Fearon and Vogelstein (1990). Germline *Apc* mutations result in the development of numerous polyps in the large intestine by the second or third decade of life of patients with FAP (familial adenomatous polyposis), the inherited, autosomal dominant predisposition to colon cancer (Kinzler *et al.*, 1991). *Apc* mutations have been identified in sporadic colorectal neoplasia as well as in inherited non-polyposis colon cancer, which results from inherited mutations in DNA mismatch repair gene.

Though loss of normal *Apc* function is an important step in the early stages of intestinal tumor formation, it is not clear how this loss contributes to tumor formation.

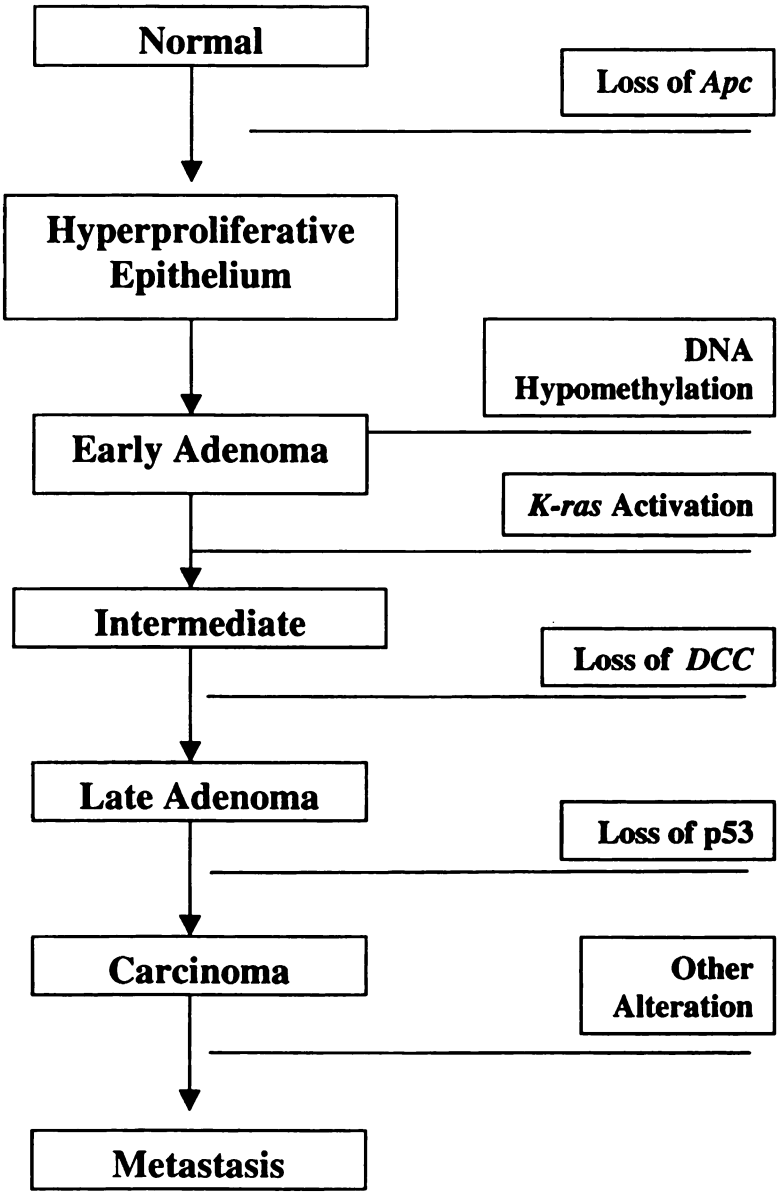

Figure 1. Multistep model for the development of colorectal carcinogenesis (Modified from Fearon and Vogelstein , 1990)

Recent findings have suggested that APC protein associates with a number of proteins including the cadherin-binding protein, β-catenin. Wild-type APC has been shown to inhibit the increased β-catenin/T cell factor (Tcf) signalling observed in colorectal cancers (Munemitsu *et al.*, 1995). The inhibitory activity of APC on β-catenin/Tcf signaling is attributed to its ability to form a macromolecular complex with β-catenin and GSK-3β (Glycogen Synthase Kinase-3β), thus facilitating the degradation of β-catenin (Munemitsu *et al.*, 1995).

Apc Mutations in Human Colon Cancers

Several hundred somatic and germline mutations have been reported in the *Apc* gene. More than 95% of both germline and somatic mutations result in premature truncation of the polypeptide. Germline mutations are distributed throughout the 5' half of the gene, and somatic mutations are concentrated in a mutation cluster region (MCR) (See Fig. 2) between codons 1286 and 1513. The high frequency of mutations in the MCR in the more severe cases of polyposis suggests that mutations in the MCR are more effective in producing tumors than mutations elsewhere in the gene. Mutations occurring before codon 157 result in a milder form of polyposis marked by fewer polyp numbers and later stage of onset. Therefore, the severity of the disease, is related to the location of the mutation and also can be modified by environmental factors and other genetic modifier loci.

Rodent Models in Colon Cancer Research

To study the effects of diet on progression from the initial mutational event to carcinoma in human subjects is nearly impossible due to the enormous genetic heterogeneity in a large group of people. In addition, there are difficulties associated with long time control of diet in these groups. Importance of good animal models to study diet and colon cancer relationships, therefore, cannot be overemphasized. In experimental work spanning nearly a century, researchers have tried to reproduce the characteristics of the human colon cancer in animal models. Most models are based on the use of carcinogenic chemicals such as dimethylhydrazine or azoxymethane. These chemicals are usually administered orally or subcutaneously to produce tumors in the intestinal tract.

Recently, the knowledge of genetic changes that accompany the morphological stages in the process of colon carcinogenesis has led to the introduction of several transgenic and knock-out mouse models. In these models either one of the tumor suppressor genes has been mutated (p53 knockout mouse) (Carbone, 1992), or a proto-oncogene has been activated [mutated *MCC* (mutated in colon cancer) mouse]. Genetic mouse models with mutated *Apc* gene include the *Apc* 1638 mouse generated by gene targeting and embryonic stem cell technology, the *Apc* (δ716) mouse, carrying a truncation mutation at codon 716 of the Apc gene (Pories *et al.*, 1993), and the Min mouse, which also carries a germline mutation in the *Apc* gene. A major advantage of using the mouse models is the ability to experimentally control the genetic background of the animal.

The Min Mouse

The Apc Min (Multiple Intestinal Neoplasia) mouse developed in the early 90's (Moser *et al*, 1990) carries a germ-line mutation in the *Apc* gene. *N*-ethyl-*N*-nitrosourea (ENU), a potent germline mutagen of the mouse, was used to induce mutations in the mice to create mouse models for various human diseases. The Min mouse was discovered upon phenotypic screening of ENU-treated mice and has become an extremely useful model for studying human intestinal cancer. The Min mouse carries an autosomal dominant mutation that has been localized to the region of mouse chromosome 18 that also carries the *Apc* gene. Sequence analysis of the *Apc* cDNA has identified the nonsense mutation resulting from a T/A → A/T transversion at nucleotide 2549 of *Apc*. The mutated gene codes for a truncated protein product that lacks the binding domains that are crucial for its function (Figure 2) (Shoemaker *et al*, 1997). The ability of the APC protein to bind and regulate β-catenin, a member of the family of intracellular catenins that regulate cell-cell adhesion by interacting with E-cadherin is thought to be the key to the involvement of this protein in tumor formation. It is believed that the truncated protein encoded in the Min mouse lacks the catenin-binding domains that are essential for regulating β-catenin binding to E-cadherin and is unable to regulate the downstream Tcf-mediated signaling pathway.

The phenotypic manifestation of the mutation in the *Apc* gene is the development of multiple adenomas throughout the intestinal tract. On the C57BL/6J genetic background, Min mice develop, on average, 50 or more tumors in the intestinal tract. Most of the tumors are benign adenomas, probably because the mice die prematurely at ~150 days of age due to secondary effects of tumor growth, such as, severe anemia and

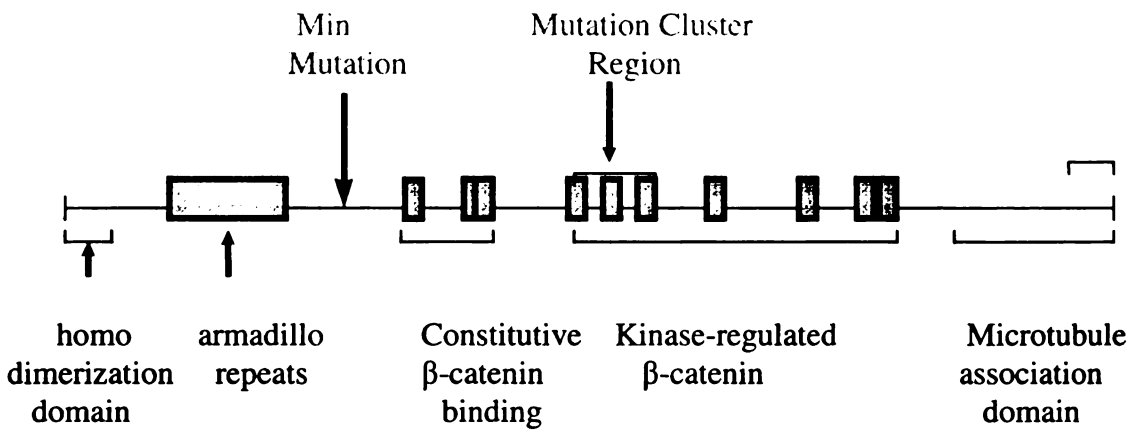

Figure 2. Potential *Apc* functional domains and location of *Apc* mutations in human colon cancer and Min mutation. (Adapted from Shoemaker *et al.*, 1997).

intestinal blockage. As described earlier, a majority of human colorectal neoplasia, both somatic and hereditary, involve loss of one or both alleles of the *Apc* gene. The Min mouse somewhat mimics the inherited form of human intestinal cancer, FAP (familial adenomatous polyposis). Though most of the tumors in FAP patients occur in the colon, the Min mouse has multiple tumors in the small intestine, and only a few in the colon.

Min Mouse in Nutrition Studies

Following the propagation of the Min mouse model in 1990, it has been used in numerous studies to study the complex relationship between dietary factors and colon cancer. Some of the dietary factors studied in the Min mouse include dietary fat, fiber, calcium, and certain phytochemicals. The results of these studies have been conflicting and at times appear to contradict the current understanding of the effect of diet in colon cancer. Diets high in animal and saturated fats are generally associated with high risk of developing colon cancer and diets rich in vegetables and fruits are associated with lower risk of colon cancer (Sandler *et al.*, 1993). However in Min mice, intake of high dietary fat and vegetable-fruit mixture had no effect on the development of intestinal tumors (Table 1) (van Kraner *et al.*, 1998). A study examining the effect of dietary fiber on intestinal tumorigenesis in the Min mouse showed no protective effects of wheat bran or resistant starch on small intestinal tumors (Pierre *et al.*, 1997). Short-chain fructo-oligosaccarides reduced tumors in the colon, but not in the small intestine where the majority of adenomas occur in Min mice (Pierre *et al.*, 1997). These results contradict numerous studies that have shown a protective effect of fibers such as wheat bran on colon cancer risk in clinical

Table 1. Studies investigating the effects of dietary factors on tumorigenesis in the Min mouse

Study	Dietary Component	Effect on tumor formation in Apc^{Min} mouse
Kraner et al, 1998	dietary fat and fruit- vegetable mixture	no effect no effect
Sorensen et al, 1998	soy isoflavones	no effect
Paulsen et al, 1997	EPA and DHA enriched fish oil	suppressed tumor growth and formation
Pierre et al, 1997	short-chain fructo-oligosaccarides	no effect on small intestinal tumors, ↓ colon tumors
Kennedy et al, 1996	soybean-derived Bowman-Birk inhibitor	↓ total tumor number

trials (Macrae, 1999), and carcinogen-induced colon carcinogenesis (Takahashi *et al.*, 1999; Compher *et al.*, 1999).

However, Kennedy *et al* (1996) found that feeding diets containing the soybean-derived Bowman-Birk protease inhibitor reduced tumor number in Min mice when the dietary treatment was started *in utero*. These studies suggest that while the effect of dietary factors is not always what is expected from current hypothesis, beginning the treatment at an earlier time point may be an important factor.

Paulsen *et al.* (1997) examined whether the n-3 polyunsaturated fatty acid ethyl ester enriched fish oil K85 (a mixture of eicosapentaenoic acid and docosahexaenoic acid as ethyl esters) could inhibit the intestinal tumorigenesis in Min mice. Dietary K85 treatment reduced the number of small intestinal tumors: in males, the maximum reduction was 66% with 0.4% of K85; and in females, the maximum reduction was 48% ($P = 0.043$) with 2.5% of K85, but the inhibition was only slightly increased from 0.4% to 2.5% of K85. The small intestinal tumor diameter was reduced by K85 in a dose-dependent manner. In the large intestine, the mean number of tumors/mouse was 1.0 +/- 0.5 in males and 0.8 +/- 0.2 in females. Although K85 treatment tended to reduce the number and diameter of the large intestinal tumors, these effects did not reach statistical significance.

Sorenson *et al.* investigated whether soy isoflavones inhibited intestinal tumor development in Min mice (Sorenson *et al.*, 1998). The mice were fed a Western-type high-risk diet (high fat, low fiber and calcium) containing two different levels (16 and 475 mg of total isoflavones per kg diet) of isoflavones from soy. No significant differences in the incidence, multiplicity, size and distribution of intestinal tumours were

observed between Min mice fed low and high isoflavone-containing diets. Thus, in contrast to epidemiological studies, their results demonstrated that high amounts of soy isoflavones present in a Western-type high risk diet were not protective against intestinal tumor development in the Min mice. The interventions that have consistently reduced tumor numbers in Min mice are the Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) such as aspirin (Barnes and Lee, 1998), sulindac (Beazer-Barclay *et al*, 1996) and piroxicam (Jacoby *et al*, 1996) (Table 1).

Non-Steroidal Anti-Inflammatory Drugs

A growing number of studies suggest that Non Steroidal Anti-Inflammatory Drugs (NSAIDs) such as aspirin and piroxicam may reduce the risk for colorectal tumorigenesis (Waddell and Loughry, 1983). These drugs have clinical application as the toxicity associated with their intake is considerably less than with other anti-cancer drugs. Several epidemiological studies have reported that a daily intake of aspirin can reduce the risk for death due to colorectal cancer. Sulindac suppressed tumorigenesis in FAP patients (Labayle *et al.*, 1994).

The NSAIDs, Piroxicam and sulindac, have been shown to significantly inhibit tumor multiplicity in Min mice. In a study where piroxicam was given to Min mice continually in the diet at a level of 200ppm beginning at 30 days of age, it reduced tumor number by a factor of 8 (Jacoby *et al.*, 1996). This effect was limited to the small intestine. The low average colon tumor number (0.6 ± 0.3) was cited as the reason why an effect in the colon was difficult to detect.

In a study by Boolbol *et al.* (1996), Min mice were given sulindac in drinking water at 160 ppm at 5-6 weeks of age. After 9 weeks of treatment the tumor number decreased from 11.9 in the untreated mice to 0.1 in sulindac treated mice. Treatment was begun after 4 weeks of age, therefore the results represent the effect of these drugs on tumor promotion and progression rather than initiation. The authors also examined the intestinal expression of cylcooxygenase-2 (Cox-2) and the levels of enterocyte apoptosis in untreated and sulindac-treated Min mice to explain the results. They found that tumor-free intestinal epithelium from Min mice expressed higher level of Cox-2 than the intestinal epithelium of normal littermates. Williams et al. (1996) also found elevated levels of Cox-2 mRNA and protein in intestinal tumors from untreated Min mice. Treatment of Min mice with sulindac reduced Cox-2 to levels observed in normal littermates. It is not certain at this point if the reduction in Cox-2 expression is responsible for the reduction in tumor multiplicity or an independent effect. Some studies suggest a prostaglandin-independent mechanism for the sulindac (Chiu *et al.*, 1997). Sulindac is a pro-drug that is modified in the liver and by colonic bacteria to its metabolically active sulfide derivative that possesses the anti-inflammatory activity.

Boolbol et al (1996) also reported a reduction in the level of enterocyte apoptosis in Min mice compared to normal littermates and a reversal of this effect by sulindac. However, this effect was more pronounced when measured by immunoperoxidase analysis and not as apparent when measured by terminal tranferase-mediated dUTP nick end-labeling (TUNEL) assay. Tumor multiplicity in control animals reported in this study was considerably lower (~12 tumors/animal) compared to the numbers reported by

other studies (~50 tumors/animal). This difference might be due to difference in diets fed or other environmental factors.

Another study reported the effect of sulindac on tumor multiplicity in Min mice (Beazer-Barclay et al., 1996). High (334 ppm) and low (167 ppm) doses of sulindac in diet and in drinking water (84 mg/L) were tested. A stronger effect of sulindac was observed when the treatment was started prenatally by treating pregnant females. This suggests that sulindac may inhibit tumor formation or induce tumor regression.

Whole Wheat and Cancer

Whole grains such as wheat are important sources of many nutrients including dietary fiber, resistant starch, oligosaccharides, trace minerals, vitamins and other compounds such as phytoestrogens and antioxidants (Slavin et al., 1999). Dietary guidelines recommend the consumption of whole grains to prevent chronic diseases. This view is supported by epidemiological studies that have shown that consumption of whole grains such as wheat, rice and corn is protective against cancer, especially gastrointestinal cancers such as gastric and colon cancer (Block, 1992; Slavin, 1994) and cardiovascular disease (Brown et al., 1985). In experimental studies, wheat bran was shown to decrease aberrant crypt foci, preserve normal proliferation and increase apoptosis in azoxymethane-induced colon cancer (Compher *et al.*, 1999). These anti-neoplastic effects were associated with increased fecal butyrate levels.

Whole wheat has several components that may protect against colon cancer. It is a rich source of fermentable carbohydrates including dietary fiber, resistant starch, and oligosaccharides. Fermentation of these undigested carbohydrates in the colon leads to

the production of short-chain fatty acids and gases. Production of short-chain fatty acids such as butyrate has been linked to a decreased risk of colon cancer. Undigested carbohydrates also increase fecal weight and decreased intestinal transit time thus limiting the exposure of intestinal epithelium to mutagenic and tumor-promoting agents such as bile acids in the fecal matter (Wrick et al., 1983).

Whole wheat also contains a number of antioxidants such as vitamins, trace minerals, and non-nutrients such as phenolic acids, lignans, phytoestrogens and phytic acid (Thompson, 1994). Whole wheat is a rich source of vitamin E. The vitamins and minerals are present in the outer layer of the grain and are lost during milling; therefore, refined flours are a poor source of these nutrients. Whole wheat is also a rich source of phenolic acids such as ferulic and caffeic acids, which are located in the bran layer. Durum wheat bran has been shown to have antioxidant properties in an *in vitro* model (Onyeneho and Hettiarchchy, 1992). Phenolic acids may also function by inducing detoxification systems, specifically the phase II conjugation reactions (Wattenberg, 1985). Phytic acid, another constituent of whole wheat, functions as an antioxidant by chelating various metals thus suppressing damaging iron-catalyzed redox reactions (Graf and Eaton, 1993). Vitamin E, another antioxidant present in whole wheat at the intracellular level protecting polyunsaturated fatty acids in the cell membrane from oxidative damage. Selenium is a trace mineral present in whole wheat that functions as an antioxidant. The amount of selenium in wheat varies and is proportional to the level of selenium in the soil in which the crop was grown. Selenium is a cofactor for the glutathione peroxidase enzyme that protects tissue against oxidative damage. Whole wheat also contains

phytoestrogens like lignans and anti-nutritive factors such as phytic acid, which may also function to reduce cancer risk (Adlercreutz, 1984).

Flax and Cancer

Interest in flaxseed as a component of diet is currently undergoing a moderate resurgence. Flaxseed contains 35% of its mass as oil, most of which is α-linolenic acid and linoleic acid (Ensminger, 1983). Flaxseed is a rich source of omega-3 fatty acids and the richest source of plant lignans. Lignans are building blocks for lignins, constituents of plant cell walls and are present in higher plants such as grains, legumes, vegetables, and seeds. The level of lignans called secoisolariciresinol diglucoside (SDG) present in flaxseed is 100-800 times higher than the amount present in other plants (Thompson, 1991). Lignans possess a dibenzyl structure that is altered when they reach the colon. Lignans are absorbed in the colon. In the liver they are conjugated with glucuronic acid or sulfate, excreted with bile, deconjugated by the colonic bacteria into mammalian lignans enterolactone and enterodiol. By virtue of their 2,3-dibenzylbutane structure, these lignans resemble potent synthetic estrogens, which may act antiestrogenically. Both enterolactone and enterodiol have been shown to express weak estrogenic and antiestrogenic properties (Adlercreutz *et al.*, 1986). Antiestrogens have displayed antitumor activity in experimental and human mammary tumors (Litherland and Jackson, 1988).

Flaxseed lignans have been shown to be inducers of β-glucuronidase activity, the enzyme responsible for the deconjugation of lignans in the colon (Jenab and Thompson, 1996). β-Glucuronidase, an inducible enzyme is also involved in the enterohepatic

circulation and activation of procarcinogens, carcinogens, mutagens and toxins that may be associated with an increased risk of colon carcinogenesis (Reddy *et al.*, 1992). Flaxseed has been shown to have beneficial effects in mammary carcinogenesis in rats when added to the diet during initiation and promotional stages (Serraino and Thompson, 1992a). In rats treated with azoxymethane, flaxseed meal supplementation of diet at 5% and 10% reduced the formation of aberrant crypts and aberrant crypt foci, probable precursors to colonic tumors, in the colon (Serraino and Thompson, 1992b).

Black Beans and Cancer

Anti-carcinogenic effects of black beans were suggested by epidemiological studies showing a low incidence of colon cancer in many Latin American countries where the consumption of dry beans is high (Correa, 1981). Dry beans are among the best known sources of dietary fiber and contain both soluble and insoluble types of dietary fibers (Hughes *et al*, 1996, Herrera *et al.*, 1998). In experimental studies, dry beans inhibited azoxymethane-induced colon carcinogenesis in rats (Hughes *et al.*, 1997). In addition, beans such as black beans are a rich source of numerous phytochemicals, including polyphenolics, that possess both anticarcinogenic and antioxidant properties.

The most notable of the phytochemicals present in the black beans is the anthocyanins. The characteristic red anthocyanin pigment found in the black bean can potentially be used as a coloring agent in foods. Anthocyanins have been shown to possess anti-oxidant properties against the potent and highly destructive peroxyl radicals (Wang *et al.*, 1997). Free radicals and their oxidants cause oxidative damage to lipids, proteins, and nucleic acids leading to cancer and atherosclerosis. The free radical

neutralizing property of these phytochemicals, such as those present in the black bean, are thought to be of central importance to prevention of cancer.

Energy Restriction and Cancer

The inhibitory effect of energy or caloric restriction on cancer has been recognized for some time. Additionally energy restriction is perhaps the only dietary treatment shown to improve longevity in laboratory animals. The association between energy restriction and cancer protection was established by work done almost a century ago. In 1909, Moreschi (Moreschi, 1909) was the first to report the effects of underfeeding on carcinogenesis. He observed that sarcoma transplanted into mice grew more slowly as less and less food was offered. In 1914, work published by Rous (Rous, 1914) showed that underfeeding also inhibited the growth of spontaneous tumors. By 1943, Tannenbaum (Tannenbaum, 1943) had shown that energy restriction inhibited the growth of both spontaneous and induced tumors. Energy restriction was shown to reduce colon tumor growth in the rats treated with azoxymethane (Pollard *et al*, 1984; Reddy *et al*, 1987). This cancer inhibitory effect of caloric restriction seems to be independent of the effect of fat in the diet. In chemically induced models of colon and mammary cancers, carcinogenesis was inhibited in 40% calorie-restricted rats even as they were fed increased percentage of dietary fat (Klurfeld *et al*, 1987). In rats, energy restriction at middle age (16 weeks) modulated the development of pre-neoplastic lesions (Lasko, 1999). Energy restriction decreased the total number of aberrant crypt foci, the precursors to colonic tumors.

Despite the abundance of literature on energy restriction and inhibition of cancer, little is known about the mechanisms underlying anti-neoplastic effects of energy

restriction. One possible mechanism suggests an effect of energy restriction on the hormonal balance in the animal. Energy restriction has been shown to stimulate the pituitary-adrenocorticotropic axis, which results in decreased levels of mitogenic and reproductive hormones (Ames and Shigenaga, 1994; Leakey *et al*, 1994; Weindruch and Walford, 1988). This energy restriction-induced switch from a reproductive pattern of life to one emphasizing repair and maintenance functions has been suggested to reduce tumor incidence by lowering the metabolic rate, hence reducing the production of toxic metabolic by-products and reducing nuclear damage. Energy restriction reduces cell proliferation and increases cell death by apoptosis in animals (Wachsman, 1996). Energy restriction was shown to reduce colon weight, the total number of cells, DNA synthesis and the total number of crypts. The reduction in the total number of cells dividing at any given time was suggested to impart resistance against induction of colon cancer to these animals (Albanes *et al.*, 1990). Up-regulation of apoptosis has also been cited as a possible mechanism by which energy restriction imparts its anti-carcinogenic effect (Wachsman, 1996). A chronic dietary restriction was shown to increase the spontaneous apoptotic rate and decrease cell proliferation rate in hepatocytes of 12-month old B6C3F1 mice (James *et al.*, 1998). Inhibition of cell proliferation and increased cell death by apoptosis may account for the anti-cancer effect of energy restriction.

Cellular proliferation and death are regulated by the expression of transcription factors that regulate cell cycling. Zhu *et al.* (1999) have studied the effects of energy restriction on expression of cell-cycle protein in chemically induced mammary carcinogenesis. Energy restriction, in a dose dependent manner, up-regulated p27/kip1, a gene product associated with cell-cycle growth arrest, and down-regulated cyclin D1, a

protein that combines with cyclin-dependent kinases to promote phosphorylation of retinoblastoma protein and the progression of cell through the cell-cycle (Zhu *et al.*, 1999). Another recent publication reported the changes in gene expression in response to aging and energy restriction (Lee *et al*, 1999). Using a novel technique called oligonucleotide based microarrays, researchers examined the expression of over 6,000 genes, as reflected by mRNA levels. The study showed that several changes in expression of genes as a result of aging, were partially reversed by energy restriction. The genes involved in energy metabolism that were down-regulated in aging animals were up regulated in energy restricted animals. Similarly, the genes involved in protein metabolism, and biosynthesis were also up-regulated by energy restriction. In contrast, the genes involved in stress response, e.g., the heat shock response, DNA-damage-inducible genes, and the oxidative genes that were up-regulated in aging animals, were down-regulated in energy restricted animals (Lee *et al*, 1999). These studies provide evidence for the hypothesis that energy restriction elicits its effects by modulating gene expression.

Complementary DNA Arrays

Oligonuceotide based microarrays or cDNA microarrays have been in use for a short time (Eggers *et al*, 1994; Fodor *et al*, 1991; Lamture *et al*, 1994), but their use is increasing in the medical research community. Microarray technology is a powerful tool for functional genomics research. Complimentary DNA arrays enable the simultaneous detection of thousands of genes in a single small sample. The microarrays consist of large numbers of cDNA molecules spotted in a systematic order on a solid substrate (such as a nylon

membrane, glass slide or silicon chip). DNA arrays are fabricated by high-speed robotics on glass or nylon substrates, for which labeled probes are used to determine complementary binding allowing parallel gene expression and gene discovery studies. Oligonucleotide microarrays are fabricated either by *in situ* light-directed combinational synthesis or by conventional synthesis followed by immobilization on glass substrates. Sample DNA is amplified by the polymerase chain reaction (PCR) and a fluorescent label is inserted and hybridized to the microarray. This technology has been successfully applied to the simultaneous expression of many thousands of genes and to large-scale gene discovery, as well as polymorphism screening and mapping of genomic DNA clones (Ramsay, 1998). The excitement surrounding microarray technology has been tempered by the limited ability of the general biomedical research community to gain access to it. Only recently, the hardware required for exploitation of the technology has become increasingly available (Bowtell, 1999).

Expression of selected genes coding for proteins with defined cellular functions has been analyzed in human cell lines derived from normal colonic mucosa, non-mucinous colonic carcinomas and mucinous colonic carcinomas (Backert et al., 1999). The expression of 10 genes was found to be altered in colon carcinoma cells. The topoisomerase II alpha and the mitosis inhibitor WEE1Hu gene were significantly suppressed in the tumor cell lines. In addition, the gene coding for the cell cycle inhibitor p21 was overexpressed only in cell lines derived from mucinous carcinomas. These alterations were confirmed by northern blotting or semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) suggesting that the cDNA array method permits a

correct identification of changes in gene expression with a relatively high accuracy (Backert et al., 1999).

The development and use of molecular-based therapy for breast cancer and other human malignancies will require a detailed molecular genetic analysis of patient tissues. In a recent study, the laser capture microdissection and high density cDNA arrays has led to the generation of gene expression profiles of cells from various stages of tumor progression as it occurs in the actual neoplastic tissue milieu. Complimentary DNA microarrays were used to monitor in vivo gene expression levels in purified normal, invasive, and metastatic breast cell populations from a single patient. The use of cDNA microarray analysis combined with laser capture microdissection has provided a powerful new approach to elucidate the *in vivo* molecular events surrounding the development and progression of breast cancer and is generally applicable to the study of malignancy (Sgroi, 1999).

The microarray analysis of gene expression profiles has been used to characterize and distinguish the mechanisms of response of colonic epithelial cells to physiological and pharmacological inducers of cell maturation. The short-chain fatty acid butyrate, produced by microbial fermentation of dietary fiber in the large intestine, is a physiological regulator of major pathways of colonic epithelial cell maturation: cell cycle arrest, lineage-specific differentiation, and apoptosis. Microarray analysis of 8,063 sequences of SW620 colonic epithelial cells upon treatment with butyrate was conducted and compared with the effects of sulindac, the nonsteroidal anti-inflammatory drug with significant chemopreventive activity for colon cancer, and curcumin. Curcumin, a component of mustard and curry is structurally and functionally related to sulindac that

also has chemopreventive activity. Although butyrate and sulindac induce a similar G0-G1 arrest, decrease in β-catenin-Tcf-LEF signaling, and apoptotic cascade, there were striking differences in the gene clusters effected by these compounds. This has important implications for characterization of chemopreventive agents and recognition of potential toxicity and synergies (Mariadason, 2000).

This potential of cDNA microarray technology in the identification of carcinogens and other environmental hazards and in determination of the relative safety of natural or synthetic chemicals to which humans are exposed is great. The use of this methodology will be critical to increase the sensitivity of detection of the potential toxic effects of environmental chemicals and to understand their risks to humans (Afshari et al., 1999).

In the past years, microarray technologies have moved beyond the proof-of-principle stage and are now being used for genome-wide expression monitoring, large-scale polymorphism screening and mapping, and for the evaluation of drug candidates (Braxton, 1998). Large-scale RNA assays and gene-expression-microarray studies are being applied at several stages in the drug-development process and could ultimately have broad applications in disease diagnosis and patient prognosis (Zweiger, 1999).

Microarray analysis has been exploited to understand the function of genes such as MYC and BRCA-1 (Coller *et al.*, 2000). MYC affects normal and neoplastic cell proliferation by altering gene expression, but the precise pathways have always remained unclear. Oligonucleotide microarrays were used to analyze expression of 6,416 genes to determine changes in gene expression caused by activation of c-MYC in primary human fibroblasts. In these experiments, 27 genes were consistently induced, and 9 genes were repressed. The identity of the genes revealed that MYC may affect many aspects of cell

physiology altered in transformed cells: cell growth, cell cycle, adhesion, and cytoskeletal organization. Identified targets included the nucleolar proteins nucleolin and fibrillarin, the eukaryotic initiation factor 5A, G1 cyclin D2 and the cyclin-dependent kinase binding protein CksHs2, cyclin-dependent kinase inhibitor p21 (Cip1), the extracellular matrix proteins fibronectin and collagen, the cytoskeletal protein tropomyosin, and tumor necrosis factor receptor associated protein TRAP1 (Coller *et al.*, 2000).

The breast and ovarian cancer susceptibility gene product BRCA1 has been reported to play an integral role in certain types of DNA repair. High density cDNA array screening of colon, lung, and breast cancer cells identified several genes affected by BRCA1 expression in a p53-independent manner, including DNA damage response genes and genes involved in cell cycle control. Notable changes included induction of the GADD45 and GADD153 genes and a reduction in cyclin B1 expression. Therefore, BRCA1 has the potential to modulate the expression of genes and function of proteins involved in cell cycle control and DNA damage response pathways (MacLachlan, 2000).

Microarrays are one of the latest breakthroughs in experimental molecular biology, which allow monitoring of gene expression for tens of thousands of genes in parallel and are already producing huge amounts of valuable data. Analysis and handling of such data are becoming major bottlenecks in the utilization of the technology (Brazma and Vilo, 2000). This shift in the biological investigation paradigm, such that the bottleneck in research is shifting from data generation to data analysis, has led to the use of hierarchical clustering, divisive clustering, self-organizing maps and k-means clustering to make sense of this mass of data (Sherlock, 2000). Microarray-based studies are uncovering broad patterns of genetic activity, providing new understanding of gene

functions and, in some cases, generating unexpected insight into transcriptional processes and biological mechanisms. One topic that has come to the forefront is how best to effectively manage and interpret the large data sets being generated. Although progress has been made, this remains a challenging opportunity for functional genomics research (Harrington, 2000).

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CHAPTER II

INHIBITION OF INTESTINAL TUMORIGENESIS IN APC^{MIN} MICE BY FOOD RESTRICTION, DEFATTED FLAX/WHOLE WHEAT AND BLACK BEANS

Mridvika, M.R. Bennink, and L.D. Bourquin

Department of Food Science and Human Nutrition, Michigan State University,
East Lansing, MI 48824-1224

ABSTRACT

Apc^{Min} (or Min) mice develop multiple adenomas in the small intestine (SI) and have been used to study the effects of diet on intestinal cancer with mixed results. We examined the effects of 20% food restriction, feeding a defatted flax/whole-wheat flour based diet and feeding a black bean-based diet for 11 weeks on tumor number and size in small intestine and colon. The NSAID drug sulindac was used as the positive control. Results: SI tumor number decreased ($P<0.05$) from 76.5 ± 5.8^{b} in the casein fed control group to 45.4 ± 5.3^{a} in the sulindac group (42% decrease), 53.2 ± 5.5^{a} in the flax/wheat group (30% decrease), 55.6 ± 5.7^{a} in the black bean group (28% decrease) and 48.9 ± 9.9^{a} in the diet restricted group (36% decrease). Sulindac reduced average SI tumor diameter from 1.32 to 1.03 mm ($P<0.01$). Black bean and flax/wheat diets increased SI tumor diameter ($P<0.05$). Colon tumor numbers did not differ significantly among groups. Black bean and flax/wheat diets increased the colon tumor volumes compared to the control group. Conclusion: black bean, flax/wheat diets and 20% dietary restriction reduced the number of adenomas in SI. However black bean and flax/wheat diets increased SI and colon tumor size. These treatments did not reduce colon tumor number.

INTRODUCTION

The Apc^{Min} (or Min: Multiple Intestinal Neoplasia) mouse is a model for human familial adenomatous polyposis (FAP), a hereditary condition in humans that progresses to colon cancer early in life if left untreated. The genetic defect is a nonsense mutation of the *Apc* gene, a gene implicated in both sporadic and inherited (e.g. FAP) human colorectal cancers (Powell *et al.*, 1992; Kinzler and Vogelstein, 1996). More than 95% of both germline and somatic mutations of the *Apc* gene result in premature truncation of the polypeptide. Functional APC has been shown to associate with more than half a dozen proteins including β-catenin and glycogen synthase kinase-3β (Su *et al.*, 1993). Wild-type APC has been shown to inhibit the increased β-catenin/T-cell factor (Tcf) signalling observed in colorectal cancers (Munemitsu *et al.*, 1995). The inhibitory activity of APC on β-catenin/Tcf signaling may be due to its ability to form a macromolecular complex with β-catenin and GSK-3β, thus facilitating the degradation of β-catenin (Rubinfeld *et al.*, 1996). The truncated APC protein in Min mice lacks the binding domains necessary to form the multimolecular complex with β-catenin and GSK-3β. Because of its central role in regulating β-catenin signalling, APC is often referred to as a gatekeeper protein. In the absence of this vital gatekeeper function, as occurs in the Min mouse, regulatory controls of the cell cycle are disabled leading to increased cell proliferation, delayed differentiation and accumulation of additional mutations. This ultimately results in the formation of multiple tumors in the colons of FAP patients and multiple adenomas throughout the intestinal tract in Min mice.

Following the introduction of the Min mouse strain (Moser *et al.*, 1990), several researchers examined the relationship between tumor development in Min mice and

dietary fat, fiber, calcium, and certain phytochemicals. Results of these studies have been inconsistent. For instance, it is generally believed that high intakes of animal and saturated fat are associated with a high risk of developing colon cancer. Conversely, diets rich in vegetables and fruits are associated with a reduced risk of colon cancer (Sandler *et al.*, 1993). However, a study examining the effects of dietary fat and a vegetable-fruit mixture on adenoma formation in Min mice (van Kraner *et al.*, 1998) found no effect of dietary fat (40% *vs* 20%) or the vegetable-fruit mixture on the development of intestinal tumors. Another study examining the effect of dietary fiber on intestinal tumorigenesis in the Min mouse showed no protective effects of wheat bran or resistant starch on small intestinal tumors (Pierre *et al.*, 1997). These results contradict numerous studies that have shown a protective effect of fibers such as wheat bran on colon cancer risk in clinical trials (Marcae, 1999) and carcinogen-induced colon carcinogenesis (Takahashi *et al.*, 1999; Compher *et al.*, 1999). Short-chain fructo-oligosaccarides reduced tumors in the colon, but not in the small intestine where the majority of adenomas occur in Min mice (Pierre *et al.*, 1997). Kennedy *et al.* (1996) observed a significant reduction in tumor number in the small intestine of Min mice fed diets containing the soybean-derived Bowman-Birk protease inhibitor. The agents that have consistently reduced tumor numbers in Min mice are the Non Steroidal Anti Inflammatory Drugs (NSAIDs) such as aspirin, sulindac and piroxicam (Barnes and Lee, 1998; Beazer-Barclay *et al.*, 1996; Jacoby *et al.*, 1996).

Energy restriction inhibits the growth of both spontaneous and induced tumors in the liver (Tannenbaum, 1940; Tannenbaum, 1943) and reduces colon tumor growth in rats treated with azoxymethane (Reddy et al., 1987). The inhibitory effect of energy

restriction on cancer development is independent of the effect of dietary fat, as chemically-induced colon and mammary cancers were inhibited by 40% energy-restriction in rats even as they were fed diets with a higher percentage of fat (Kritchevsky *et al.*, 1984; Klurfeld *et al.*, 1987). Anti-carcinogenic effects of black beans were suggested by epidemiological studies showing a low incidence of colon cancer in many Latin American countries where the consumption of dry beans is high (Correa, 1981). Beans such as black beans are a rich source of numerous phytochemicals, including polyphenolics, that possess both anticarcinogenic and antioxidant properties (Takeoka *et al.*, 1997; Griffiths, 1981). In addition, dry beans are among the best known sources of dietary fiber and contain both soluble and insoluble types of dietary fibers (Hughes *et al.*, 1996; Herrera *et al.*, 1998). In experimental studies, dry beans inhibited azoxymethane-induced colon carcinogenesis in rats (Hughes *et al.*, 1997). Flaxseed, a rich source of mammalian lignan precursors such as secoisolariciresinol diglycoside, has been shown to have protective effects in mammary (Serraino and Thompson, 1992a) and colon carcinogenesis (Serraino and Thompson, 1992b; Jenab and Thompson, 1996). Colonic bacteria convert plant lignans, such as those found in flaxseed, into mammalian lignans, mainly enterolactone and enterodiol. These compounds have been suggested to possess anti-proliferative activity (Herano *et al.*, 1990). Whole-wheat flour was added to the defatted flax diet to improve the amino acid score of this diet. Wheat also contains phytochemicals with antioxidant properties such as phytic acid and phenolic compounds (Slavin et al., 1999).

The present study examined the effect of feeding four diets on tumor number and size in the small intestine and colon in the Min mouse. The dietary treatments were: food

restriction (FR), black bean (*Phaeolus vulgaris*) diet (BB), defatted flax/whole-wheat flour diet (FW), a control group (casein diet) (CT) and positive control group that received 200 ppm sulindac in drinking water (SL). These diets have been shown in epidemiological studies and initiation-promoter models to have a protective role in colon cancer. Our objective was to determine the anti-neoplastic effects of these dietary factors in Min mice.

MATERIALS AND METHODS

Experimental Design- Min mice were obtained from a breeding colony maintained on site by crossing C57Bl/6J Min male carrier mice with normal C57Bl/6J females. Mice were not genotyped before assignment to treatment. Based on 50% incidence of carriers in progeny and target of 25 Min mice per treatment group, a total of 50 mice (carriers and non-carriers) were assigned to each treatment group. The actual number of Min mice for each treatment was between 21 and 26. Distribution of males and females between treatments was kept as even as possible, except the food restriction group had 20 males and 2 females. Mice were weaned at 4 weeks of age, assigned to dietary treatments in the fifth week and randomly assigned to one of these treatment groups: control (CT), food restriction (FR), black bean (BB), defatted flax/whole-wheat (FW) or sulindac (SL) group. Mice were weighed weekly. The dietary treatment period was 11 weeks, after which mice were killed by carbon-dioxide asphyxiation and the number of tumors in small intestines and colons were counted.

Animal housing- Mice were housed in a temperature (70-72 F) and humidity (40-70%) controlled room in plastic cages in accordance with NIH, USDA and university guidelines for laboratory animal research. All mice were housed in plastic cages. Mice in the FR treatment group were housed individually. All other mice were housed 2-4 mice/box with same sex littermates.

Animal diets- All treatment groups were fed powdered diets that were similar in macro-nutrient composition (approximately 16.7% fat, 18.9% protein, 20% fiber). The control

Table 1. Composition of the control, black bean and defatted flax/whole-wheat diets.

Ingredient	*Control*	*Black Bean*	*Defatted Flax/ Whole-Wheat*
	g/kg	g/kg	g/kg
Casein	222.2	-	-
Black bean flour	-	711.1	-
Wheat flour	-	-	350
Defatted flax flour	-	-	400
Sucrose	355	-	51.9
Soybean oil	166.7	156.0	142.3
Solka-floc fiber	200	76.8	-
Mineral mix (AIN-93G-MX)	38.89	38.89	38.89
Vitamin mix (AIN-93-VX)	11.11	11.11	11.11
L-Cysteine	3.33	3.33	3.33
Choline bitartarate	2.78	2.78	2.78
Tert-butylhydroquinone	0.02	0.02	0.02

diet was a modified AIN 93-G diet with a higher fat (16.7%) and fiber (20%) content than the standard AIN-93G diet (Table 1). Diet ingredients were purchased from Dyets Inc. (Bethlehem, PA). Defatted flax flour and whole-wheat flour were combined to provide all of the essential amino acids for mice. All diets except the FW diet had additional fiber (Solka floc) added to provide the same fiber content as the FW diet. To obtain the black bean flour, the beans were soaked overnight, cooked at 90-95 °C until soft, dried at 50-55 °C and ground to a meal. Sulindac (Sigma Chemical Company, St. Louis, MO) was added to the drinking water (200 mg/L; pH 7) of mice in the positive control group (SL). For the FR treatment group, the amount of the control diet consumed by mice was estimated in a pilot study where mice were housed in wire bottom cages and food disappearance and food spillage were measured. Based on these results, the FR male and female mice were fed 3.0 and 2.5 grams of control diet each day, which corresponded to approximately 80% of *ad libitum* consumption.

Tissue Preparation- The small intestines and colons were opened longitudinally, emptied of their contents, and rinsed with water. The ceca were not fixed or examined for this experiment. The colons were pinned flat and the small intestines were placed on a sheet of filter paper and pressed flat with a glass rod. A second sheet of filter paper was placed over the flattened small intestines. Both small intestines and colons were fixed overnight in 10% neutral buffered formalin. The tissues were stained in a solution of 0.2% methylene blue in water and stored in 1% formalin in phosphate buffered saline (PBS) until neoplasms were counted. Tumor number and tumor diameter in the small intestine were measured under a dissecting microscope at 15x magnification. In the

colon, only raised tumors were counted and three-dimensional diameters measured. An average tumor diameter was computed to calculate tumor volume, $(\pi \times dia^3)/6$.

Statistical Analysis- The data were analyzed by two-way analysis of variance (treatment x sex). When there was a significant treatment effect ($P<0.05$), multiple comparisons were conducted using the Least Significant Difference method. The colon tumor number data was analyzed with Wilcoxon's Rank Sum Test as the data were not normally distributed.

RESULTS

Three female and 1 male mice in the BB group died during the 11 week dietary treatment period. The tumor data from these animals were not collected and hence is not included in the results.

Growth The maximum weight gains over the 11-week study period are reported in Figure 1. Maximum weight gain was defined as: maximum weight during the study period – initial weight at the beginning of the study. The difference in weight gain between males and females was not statistically significant. As expected, the weight gain by the FR group, fed approximately 20% less food compared to the CT group, was significantly lower than the CT group. The weight gains for animals in BB, FW and SL groups were not significantly different from animals in the CT group.

Small Intestinal Adenomas There was a strong trend for female mice to have more adenomas than male mice ($P<0.0545$)(Table 2). There was a trend for female mice to have smaller adenomas than males (Table 2). Mice in the CT group had an average of 76.5 ± 5.8 small intestinal adenomas (Table 3). The SL group (positive control) had 42% fewer adenomas than the CT group whereas treatments FW, BB and FR reduced small intestine tumor number by approximately 30%, 28% and 36%, respectively. These reductions in small intestinal tumors were highly significant ($P<0.005$). Average adenoma diameter observed in the SL group was significantly smaller than that found in the CT group, whereas adenoma diameters were significantly larger in the FW and BB groups compared to the CT group. Small intestinal tumor diameter was unchanged by FR.

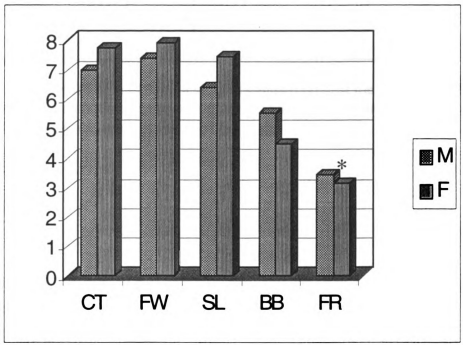

Figure 1. Weight gain in mice in treatment groups control diet (CT), defatted flax/ whole wheat diet (FW), sulindac (SL), black bean based diet (BB) and feed restricted diet (FR). The weight gain in FR group was significantly lower than the CT group ($P<0.05$). The weight gains in other four groups were statistically similar.

Table 2. Overall gender effects in SI and colon adenoma numbers and sizes in min mice[1].

	Female (n=49)	*Male (n=63)*	*P Value*
SI Adenoma Number	61.7 (4.9)	50.1 (3.5)	0.0545
SI Adenoma Diameter (mm)	1.41 (0.04)	1.49 (0.03)	0.0804
Colon Adenoma Number	1.2 (0.33)	1.4 (0.23)	0.6020
Colon Adenoma Volume (mm^3)	3.19 (1.08)	3.93 (0.77)	0.5727

Data are expressed as least square means (standard error of the means). Differences in carcinogenic parameters between male and female mice were analyzed with two way analysis of variance.

Table 3. Small intestinal tumor number and diameter in Min mice fed the control diet and treatment diets[1].

Treatment	*n*	*SI tumor number*	*SI Tumor size*
Control	21	76.5 (5.8)[b]	1.32 (0.04)[b]
Sulindac	26	45.4 (5.3)[a]	1.03 (0.04)[a]
Black bean	22	55.6 (5.7)[a]	2.08 (0.04)[d]
Defatted flax	24	53.2 (5.5)[a]	1.65 (0.04)[c]
Food restricted	22	48.9 (9.9)[a]	1.22 (0.04)[b]

[1]Data are expressed as least square means (SEM). Differences between treatments were analyzed by two-way analysis of variance followed by Least Significant Difference test for multiple comparisons. Means in a column with different superscripts denote significant differences ($P<0.05$).

Colon Adenomas The number of adenomas was not statistically different between male and female mice (Table 2). The number of colonic adenomas ranged from 0-3 per mouse and there was no significant treatment effect on colon tumor number ($P>0.05$). (Table 4). The adenomas in the FW-fed group were significantly larger compared to the SL group ($p<0.05$) and the adenomas in the BB group were larger than in the SL and CT groups ($p<0.05$).

Table 4. Colon tumor number and volume in Min mice fed the control and treatment diets.

Treatment	*n*	*Colon tumor number*	*Colon Tumor volume* (mm^3)
Control	21	1.0 (0.4)	2.74 (1.29)^{ab}
Sulindac	26	2.1 (0.3)	0.48 (1.16)^{a}
Black bean	22	1.2 (0.4)	7.20 (1.27)^{c}
Defatted flax	24	1.1 (0.4)	5.69 (1.22)^{bc}
Food restricted	22	1.2 (0.4)	1.69 (2.19)^{a}

Data are expressed as least square means (SEM). There were no significant differences in colon tumor numbers (Wilcoxon's Rank Sum test, $P<0.05$). Colon tumor volume were calculated using the formula $v=(\pi \times \text{diameter}^3)/ 6$. The colon tumor volumes were analyzed using one-way analysis of variance followed by Least Significant Difference test. Means in a column with different superscripts denote significant differences ($P<0.05$).

DISCUSSION

An inverse relationship between the consumption of NSAIDs and intestinal cancer has been observed in both human epidemiological studies and animal tumor models. In humans, regular use of NSAIDs has been associated with a 40-50% decrease in colon cancer incidence (Thun *et al.*, 1991; Giovanucci *et al.*, 1995). Administration of 334 ppm sulindac in drinking water decreased intestinal tumor number in Min mice by 38% (Beazer-Barclay *et al.*, 1996). The 38% reduction in tumor number reported by Beazer-Barclay *et al.* is in agreement with the 42% reduction we observed. Some researchers have reported a much higher level of tumor reduction (90%) with sulindac (Boolbol *et al.*, 1996). The observations that Min mice have elevated levels of cyclooxygenase-2 (Cox-2), an enzyme involved in prostaglandin (PG) metabolism, and a decreased fraction of enterocytes undergoing apoptosis may explain tumor inhibition by NSAIDs. Sulindac has been shown to block the overexpression of Cox-2 and to restore apoptosis in Min mice (Boolbol *et al.*, 1996). However, inhibition of PG biosynthesis is not required to observe an antitumor effect with sulindac. Piazza *et al.* (1995) have shown the inhibition of azoxymethane-induced colon carcinogenesis with sulindac sulfone, a metabolite of sulindac that lacks Cox inhibitory activity. In Min mice, regression of pre-existing tumors has also been shown to be independent of prostaglandin biosynthesis (Chiu *et al.*, 1997). Both sulindac sulfone and sulfide have been shown to induce apoptosis in HT-29 human colon carcinoma cells, suggesting that induction of apoptosis might be responsible for anti-neoplastic effect of sulindac (Piazza et al., 1997).

The 28% reduction in small intestine tumor number by the black bean diet is consistent with epidemiological data (Correa, 1981) and a rat tumor study (Hughes *et al.*,

1997). Persons in Latin American countries, where dry beans such as black beans are a staple, have a low incidence of colon cancer (Correa, 1981). Hughes *et al.* (1997) observed an inhibition of azoxymethane-induced colon cancer in rats fed pinto beans. The effect of feeding dry beans on adenoma formation in Min mice has not previously been reported. Black beans contain a number of factors that have traditionally been studied by nutritionists because they interfere with protein digestibility and availability and trace mineral bioavailability. Current research suggests that these anti-nutritive factors may have anti-cancer properties. Certain foods, especially colorful fruits and vegetables, contain a variety of compounds such as flavones, flavanones, anthocyanins and catechins that may offer antioxidant protection against free radicals that can cause oxidative damage which may be an important factor in the development of cancer (Ames and Shigenaga, 1994). Black beans are a rich source of the intense red anthocyanin pigments (conc. 213$\pm$2 mg/100 g bean), which occur primarily in the skin of the black beans (Takeoka *et al.*, 1997). The potent antioxidant properties of anthocyanins against the peroxyl radical have been confirmed (Wang *et al.*, 1997). Anthocyanins from tart cherries have been shown to inhibit adenoma formation in Min mice (Kang *et al.*, 2000). Black beans also contain polyphenols such as tannins (Griffiths, 1981) and tannins have been shown to possess anticarcinogenic properties in several *in vitro* studies. Despite a decrease in SI adenoma number in response to BB diet in our study, the largest SI and colon tumors were seen in the BB group. We observed that mice with large tumors in the small intestine had extensive bleeding from the centers of the tumors. The large adenomas might explain the death of four mice in the BB treatment group.

Flaxseed has been shown to possess anti-tumorigenic properties in carcinogen-induced mammary tumors (Thompson *et al.*, 1996). Rats fed a diet containing 2.5% defatted flax flour had a significant reduction in the number of aberrant crypt foci in the colon (Jenab and Thompon, 1996). Defatted flaxseed is a rich source of mammalian lignan precursors such as secoisolariciresinol diglycoside (Thompson *et al.*, 1991) that can yield mammalian lignans (enterodiol and entrolactone) by the action of the colonic bacteria. These molecules closely resemble synthetic estrogenic compounds and their anti-tumor activity may be due to estrogenic/anti-estrogenic effects. Additionally, the lignan secoisolariciresinol diglycoside has been shown to have hydroxyl radical-scavenging properties in an *in vitro* system (Prasad, 1997). The reduction in tumor number in the small intestine in the FW group may also be due to the presence of antioxidant phytochemicals in whole-wheat flour. Wheat is a source of phenolic acids, which are primarily concentrated in the bran fraction of the wheat. Phenolic acids present in wheat have been shown to possess antioxidant properties in an *in vitro* assay (Oneyeneho and Hettiarachy, 1992). In our experiment, feeding the FW diet led to a reduction in the number of small intestinal adenomas formed but the diet also resulted in a significant increase in the small intestinal adenoma size. This suggests that eating defatted flax and wheat flour inhibited the formation of adenomas but enhanced the growth of adenomas once the adenoma became established. The precise mechanism for this effect is not clear, however, these diets may contain hitherto unrecognized pro-angiogenic factors that may facilitate tumor growth.

Energy restriction has been shown to inhibit the growth of spontaneous tumors (Rous, 1914) and induced tumors (Tannenbaum, 1943). Energy restriction was also

shown to reduce colon tumor growth in rats treated with azoxymethane (Pollard *et al.*, 1984; Reddy *et al.*, 1987). This cancer inhibitory effect of caloric restriction appears to be independent of the effect of fat in the diet. In chemically induced models of colon and mammary cancers, carcinogenesis was inhibited in 40% calorie-restricted rats even though they were fed diets with higher percentages of fat compared to *ad libitum* fed control animls (Klurfeld et al., 1987).

The mechanisms underlying the effects of energy restriction are poorly understood. Food restriction has been shown to stimulate the pituitary-adrenocorticotropic axis, which results in decreased levels of mitogenic and reproductive hormones (Leakey et al., 1994;Weindruch and Walford, 1988). This energy restriction-induced switch from a reproductive pattern of life to one emphasizing repair and maintenance has been suggested to reduce tumor incidence by reducing the production of toxic metabolic by-products and reducing nuclear damage. Furthermore there is evidence that cell proliferation is decreased (Albanes, 1990) and cell death by apoptosis is increased in energy restricted animals (Wachman, 1996; James *et al.*, 1998). This energy restriction-induced increase in cell death by apoptosis coupled with a decrease in proliferation could explain the reduction in adenoma formation observed in our study.

Energy restriction can alter gene expression. Zhu *et al.* (1999) reported that energy restriction, in a dose dependent manner, up-regulated p27/kip1, a protein associated with cell cycle growth arrest, and down-regulated cyclin D1, a protein that promotes the progression of cells through the cell cycle implying that energy restriction may inhibit cell proliferation. In addition, Lee *et al.* (1999) have shown the energy restriction-induced change in gene expression of hundreds of genes involved in aging.

Their study showed that changes in gene expression as a result of aging, particularly the genes involved in energy metabolism, protein metabolism, biosynthesis, and stress response, were partially reversed by energy restriction (Lee *et al.*, 1999). These studies provide evidence that energy restriction elicits its effects by modulating gene expression. Energy restriction may inhibit tumor promotion or progression by modulating expression of oncogenes or tumor suppressor genes. In the Min mouse, which carries one mutated *Apc* allele, food restriction significantly reduced the formation and growth of adenomas, suggesting that food restriction modulates promotion and progression of carcinogenesis. As a heterozygous *Apc* mutation by itself is not enough for the formation of adenomas and since further mutations must occur for tumors to form, energy restriction must influence the occurrence of these subsequent mutational events.

Previous to the present study, the Min mouse model had been used in colon cancer-diet studies with mixed results. In this study, we clearly demonstrate a significant effect of diet on adenoma formation in the small intestine in mice heterozygous for mutated *Apc* gene. Feeding a defatted flax/ whole-wheat flour, a black bean flour-based diet, or a 20% diet restriction reduced the number of adenomas in the small intestine of Min mice by approximately 28%. The colon tumor number was not influenced by the dietary treatments tested in this study. This could be due to the very low number of adenomas formed in the colon. The shortened life span of these mice due to small intestinal bleeding might be responsible for the low numbers of colon adenomas seen in this model. Colon tumorigenesis is probably affected by factors not operative in the small intestine such as fermentation products in fiber-rich diets. These potential effects were probably not detected in this study due to model limitations.

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CHAPTER III

ENERGY RESTRICTION INHIBITS TUMORIGENESIS AND ALTERS GENE EXPRESSION IN APC^{MIN} MICE.

Mridvika[1], M.R. Bennink[1], M. Fielden[2], and T. Zacharewski[2]

Departments of Food Science and Human Nutrition[1] and
Biochemistry and Molecular Biology[2],
Michigan State University, East Lansing, MI.

ABSTRACT

The Apc^{Min} (+/-) (Min) mice carry a germ-line mutation in the APC gene, and develop multiple adenomas in the small intestine early in life. Inhibition of intestinal tumorigenesis in Min mice after 11 weeks of 20% food restriction has been shown previously (Mridvika and Bennink, 2000). The objectives of this study were: 1) to determine effects of feeding 30% energy restricted (30% fewer calories from carbohydrates) vs. control diet (modified AIN-93) for 3, 6 and 10 weeks on adenoma growth and formation in Min mice, and 2) to determine effects of 30% energy restriction for 10 weeks on gene expression in adenoma tissue of Min mice. Adenoma number and size in the small intestine and colon were measured to determine adenoma formation and growth. Complimentary DNA based arrays were used to determine expression of 1,200 mouse genes in small intestinal tumor tissue of control and energy restricted mice. The number and diameter of adenomas in the small intestine in the restricted group at times 3, 6 and 10 weeks were significantly lower than in the control group ($P<0.05$). In the colon, the changes in adenoma number and volume induced by energy restriction were not different compared to controls ($P>0.05$). Of 1,200 genes studied, 8 were overexpressed and 13 were underexpressed in adenoma tissue of energy restricted mice compared to *ad libitum* fed mice. Four of the underexpressed genes were oncogenes and 4 of the overexpressed genes were immune function proteins. Conclusions: Energy restriction reduces adenoma number and size in the small intestine in the Min mouse but does not cause a significant change in adenoma number and size in the colon. The reduced adenoma number and size were associated with changes in expression of 21 genes.

INTRODUCTION

The effects of energy restriction on carcinogenesis have been recognized for some time. In 1909, Moreschi was the first to report the effects of underfeeding on carcinogenesis. He observed that sarcoma transplanted into mice grew more slowly as less and less food was offered. In 1914, Rous and Tannenbaum showed that underfeeding also inhibited the growth of spontaneous tumors. By 1943, Tannenbaum had shown that energy restriction inhibited the growth of both spontaneous and induced tumors. Forty years later three groups demonstrated that energy restriction reduced the growth of azoxymethane-induced colon tumors in rats (Pollard *et al.*, 1984; Klurfeld *et al.*, 1987; Reddy *et al.*, 1987). This inhibition of cancer by calorie restriction appears to be independent of the proportion of calories derived from dietary fat. In chemically induced models of colon and mammary cancers, carcinogenesis was inhibited in 40% calorie-restricted rats even though they were fed a diet containing a higher percentage of calories from fat (Klurfeld *et al.*, 1987).

Despite the abundance of evidence for an inhibitory effect of energy restriction on cancer, the mechanisms underlying this effect are poorly understood. Several mechanisms have been postulated to explain this effect, such as, increased DNA repair activity, altered gene expression, depressed metabolic rate and reduced oxidative stress. Other proposed mechanisms suggest that energy restriction elicits its effects by altering the hormonal balance in the animal. Energy restriction has been shown to stimulate the pituitary-adrenocorticotropic axis, which results in decreased levels of mitogenic and reproductive hormones (Weindruch and Walford, 1988; Ames and Shigenaga, 1994; Leakey *et al.*, 1994). This energy restriction-induced switch from a reproductive pattern

of life to one emphasizing repair and maintenance functions has been suggested to reduce tumor incidence by lowering the metabolic rate, hence reducing the production of metabolic by-products that could cause nuclear damage. There is evidence that cell proliferation is decreased, and cell death by apoptosis is increased, in energy restricted animals (Albanes *et al.*, 1990; James *et al.*, 1998). Energy restriction reduced colon weight, the number of cells engaged in DNA synthesis, and the total number of crypts. The reduction in cell divion at any given time was suggested to impart resistance against induction of colon cancer in these animals (Albanes *et al.*, 1990). A chronic dietary restriction was shown to increase the spontaneous apoptotic rate and to decrease cell proliferation rate in hepatocytes of 12-month old B6C3F1 mice (James *et al.*, 1998).

Cellular proliferation and death are controlled by the expression of genes that regulate cell division cycle. Energy restriction has been shown to alter gene expression. Zhu *et al.* (1999) have studied the effects of energy restriction on expression of genes that are involved in cell cycle progression in chemically induced mammary carcinogenesis. Energy restriction, in a dose dependent manner, up-regulated p27/kip1, a gene product associated with cell cycle growth arrest, and down-regulated cyclin D1, a protein that combines with cyclin-dependent kinases to promote phosphorylation of retinoblastoma protein and progression of the cell through the cell cycle. Lee *et al.* (1999) have reported changes in gene expression in mouse muscle cells in response to aging and energy restriction. Their study showed that age-related changes in gene expression were partially reversed by energy restriction. For example genes involved in energy metabolism were down-regulated in aged animals but were up regulated in energy restricted animals. Similarly, genes involved in protein metabolism and biosynthesis were up-regulated as a result of energy restriction. In contrast, the genes involved in stress

response, e.g., the heat shock response genes, DNA-damage-inducible genes, and oxidative genes were up-regulated in aged animals, but were down-regulated in energy restricted animals (Lee *et al*, 1999). These studies provide evidence for the modulation of gene expression by energy restriction. The effect of energy restriction on expression of a wide range of genes in the Min mouse model has not been reported.

In the Min mouse, a model for human colon cancer, a germline mutation in a single allele of the *Apc* gene leads to formation of multiple adenomas in the small intestine. Tumors appear in the small intestine of Min mouse very early in life with intestinal adenomas present as early as 3 weeks after birth. Tumor number increases with age and plateaus at about 12 weeks after birth (Kang et al, 1999). However, the focal nature of adenomas and the presence of tumor-free regions in Min mice suggest that a single *Apc* mutation is not sufficient for tumor formation and further mutations are required to form an adenoma. We reported earlier that 20% energy restriction for a 11-week period significantly reduced the number of adenomas in the small intestine of Min mice. Thus, energy restriction may reduce adenoma number by delaying or preventing the occurrence of subsequent mutational events. The present study examined adenoma formation and development in Min mice after 3, 6 and 10 weeks of 30% energy restriction. In addition, complimentary DNA-based array technology was used to identify genes whose expression was altered by energy restriction.

MATERIALS AND METHODS

Experimental design

Fifty-six female Min mice were assigned to the 30% energy restricted (ER) or *ad libitum* fed control (CT) groups at 3-5 weeks of age. Eight mice from each group were killed at 3, 6 and 10 weeks after treatment was begun and tumor data were collected. Two of the mice from each group killed after 10 weeks of treatment had total RNA collected for cDNA microarray analysis. Body weight were measured weekly during the 10 week trial.

Animal Diets

Feed intake in the CT group was measured twice weekly and feed intake for the restricted group was adjusted accordingly to provide 70% of the calories consumed by CT group. All mice were individually housed in wire bottom cages to facilitate measurement of feed intake. The diet for the energy restricted group was formulated to be lacking only in energy from carbohydrates (cornstarch). The energy restricted group and the *ad libitum* fed group received an equal amount of all other nutrients (Table 1).

Tissue Preparation

Mice were killed by CO_2 inhalation, and small intestines and colons were removed. The small intestines and colons were cut open longitudinally and cleaned with water. The colons were pinned flat, the small intestines were flattened between sheets of filter paper, and both were fixed overnight in 4% 4 buffered formalin (pH 7.4). The tissues were stained in a 0.2% solution of methylene blue and stored in 1% formalin in phosphate buffered saline until the tumors were counted and measured. Counting and measuring of

Table 1. Composition of diets fed to control and energy restricted groups and the mean daily intake of each component.

Diet Ingredients	Ad Libitum Diet		Calorie-Restricted Diet	
	g/kg	Mean daily intake (g)	g/kg	Mean daily intake (g)
Cornstarch	399.4	1.28	279.6	0.7
Casein	222.2	0.71	222.2	0.6
Sucrose	100	0.32	100	0.25
Soybean oil	166.7	0.53	166.7	0.42
Solka floc	55.5	0.18	55.5	0.13
Mineral mix (AIN-93G-MX)	38.89	0.12	38.8	0.09
Vitamin mix (AIN-93-VX)	11.11	0.04	11.11	0.03
L-Cysteine	3.33	0.01	3.33	0.01
Choline bitartarate	2.78	<0.01	2.78	<0.01
T-butylhydroquinone	0.02	<0.01	0.02	<0.01
Calories/g	4.25		4.28	
Calories consumed/day		13.6		9.4

tumors were done by a person blinded to the treatments. To harvest RNA for microarray analysis, small intestines were opened longitudinally, emptied of their contents and rinsed in water. The whole tissue was examined under a dissecting microscope to identify tumors. Tumor tissue was separated from the underlying muscle and connective tissue layer with the tip of a pasteur pipette and dispensed into microfuge tubes containing Trizol LS reagent (Gibco BRL, Rockville, MD). Trizol LS reagent is a mono-phasic solution containing phenol and isothiocyanate. RNA was isolated by the single step RNA isolation method developed by Chomczynski and Sacchi (1987). In this experiment, the tumors from animals in each treatment group were pooled to ensure sufficient amounts of RNA.

RNA Isolation

Tumor tissues from control and energy restricted mice were lysed in Trizol LS reagent by sonication to ensure complete dissociation of nucleoprotein complexes. Total RNA was obtained by following the instructions for the use of Trizol reagent provided by Gibco BRL (Rockville, MD). The clear RNA pellet was washed in ethanol:water solution and dissolved in RNase-free water.

cDNA Synthesis and Hybridization

Five micrograms of mRNA was used to synthesize cDNA using MMLV reverse transcriptase (Gibco BRL, Rockville, MD) and oligo dT primer. The membranes are pre-hybridized with ExpressHyb (Clontech, Palo Alto, CA) and salmon sperm DNA for 30 minutes at 68°C. Non-specific binding was reduced by the addition of 5 μg of human C_0t

DNA to the hybridization mixture. The [^{33}P]dATP labeled probes from tumor tissue from energy restricted and control animals were hybridized to pre-wet membranes overnight at 42 °C in ExpressHyb solution. The membranes were washed 4x30 minutes with 2xSCC/1% SDS at 68 °C and 2 washes with 2xSCC/0.5%SDS also at 68 °C. To detect the [^{33}P] label, the phosphor screen was exposed from 1 hour to overnight. The label was visualized and quantitated using a phosphorimager and comparisons of gene expression across groups were made.

Numerical data was normalized by dividing each value by the overall mean of values derived from that membrane. Fold expression was obtained by taking ratios of the values for the two treatments.

RESULTS

Growth

The growth of the *ad libitum* fed and energy restricted mice are shown in Figure 1. The weights of the restricted mice were consistently and significantly lower than those of the *ad libitum* fed mice. The *ad libitum* fed control mice gained weight steadily during the first eight weeks of treatment and then began to lose weight during the final two weeks of the treatment. The restricted mice maintained their weight during the first 4 weeks and gained weight slightly in the following six weeks.

Small Intestinal Adenoma number

The number of adenomas increased during the first six weeks for both treatment groups. The increase, however, was more pronounced in the *ad libitum* fed control mice. The number of adenomas in the 30% energy restricted group at 3, 6 and 10 weeks was statistically less than the number of adenomas in the control group ($P<0.05$) (Figure 2). The energy restricted group had 33% fewer tumors than the *ad libitum* fed mice.

Small Intestinal Adenoma Diameter

Changes in small intestinal adenoma diameter at 3, 6 and 10 weeks in the control and restricted groups are shown in Figure 3. There was a steady increase in the diameter of the adenomas in the control group from 0 to 10 weeks, whereas, the adenoma diameter in energy restricted mice remained unchanged. Overall treatment effect was significant at $p<0.05$. Effect of time, however, was not significant.

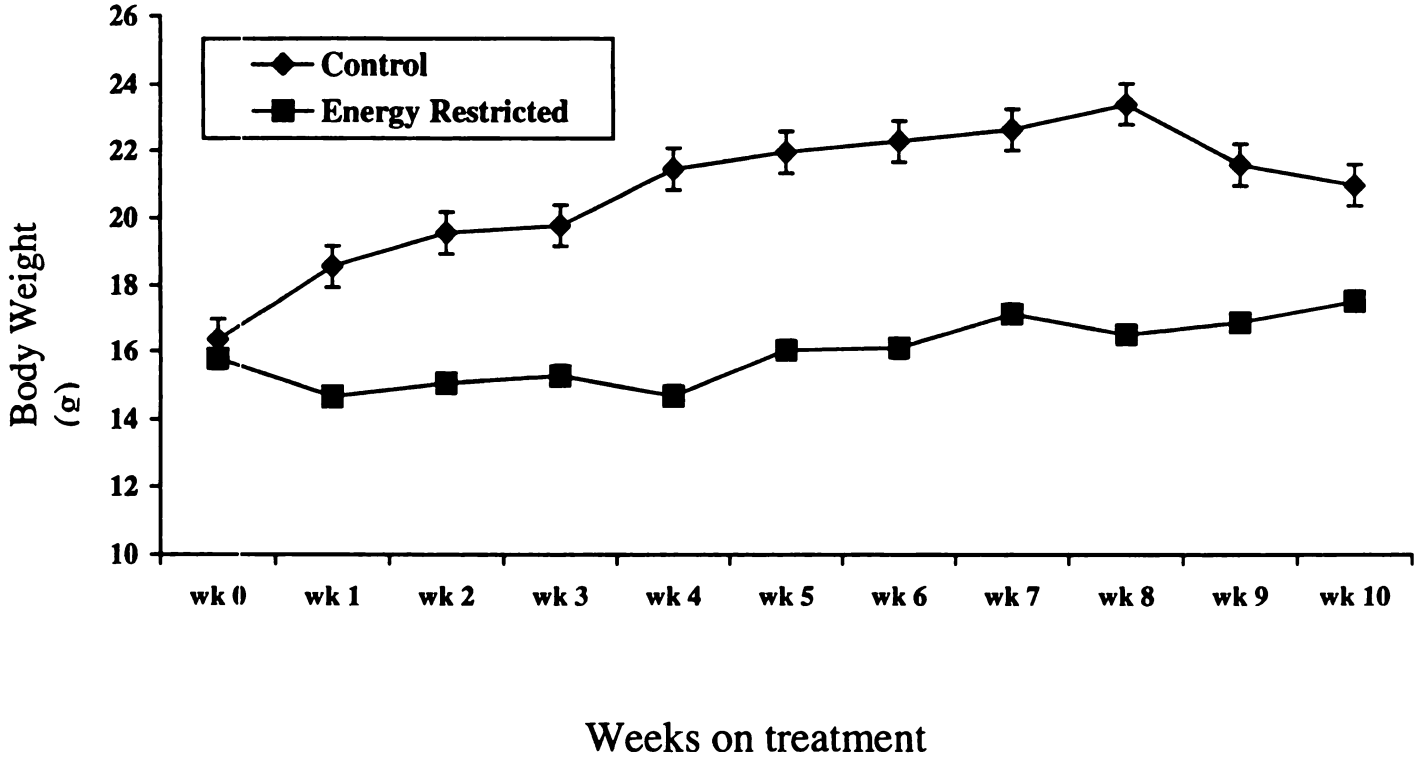

Figure 1. Body weight of *ad libitum* fed control mice and 30% energy restricted mice over the 10 week study period.

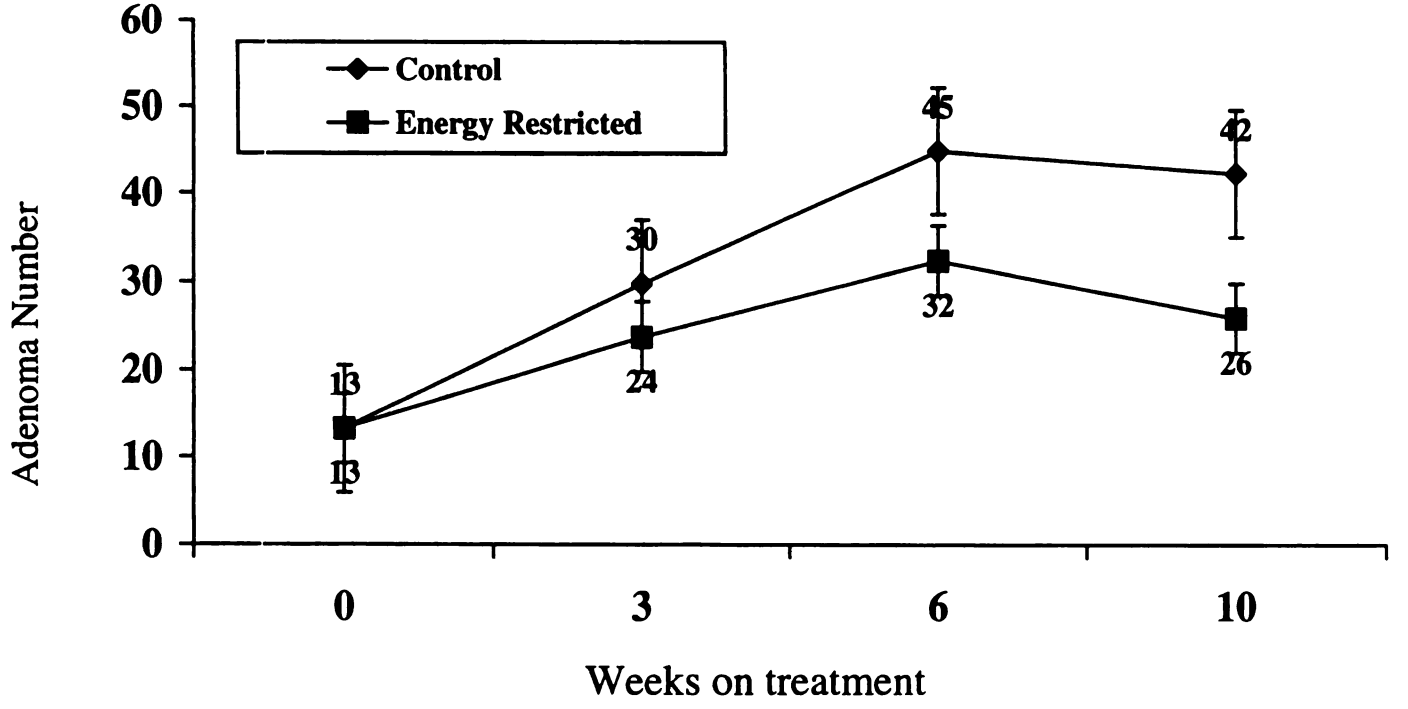

Figure 2. Small intestine adenoma numbers in *ad libitum*-fed control mice and 30% energy restricted mice at 0, 3, 6 and 10 weeks of treatment. The bars represent SEM. The adenoma numbers for *ad libitum*-fed control group were significantly higher than adenoma numbers for the energy restricted group at 6 and 10 weeks of treatment($P<0.05$).

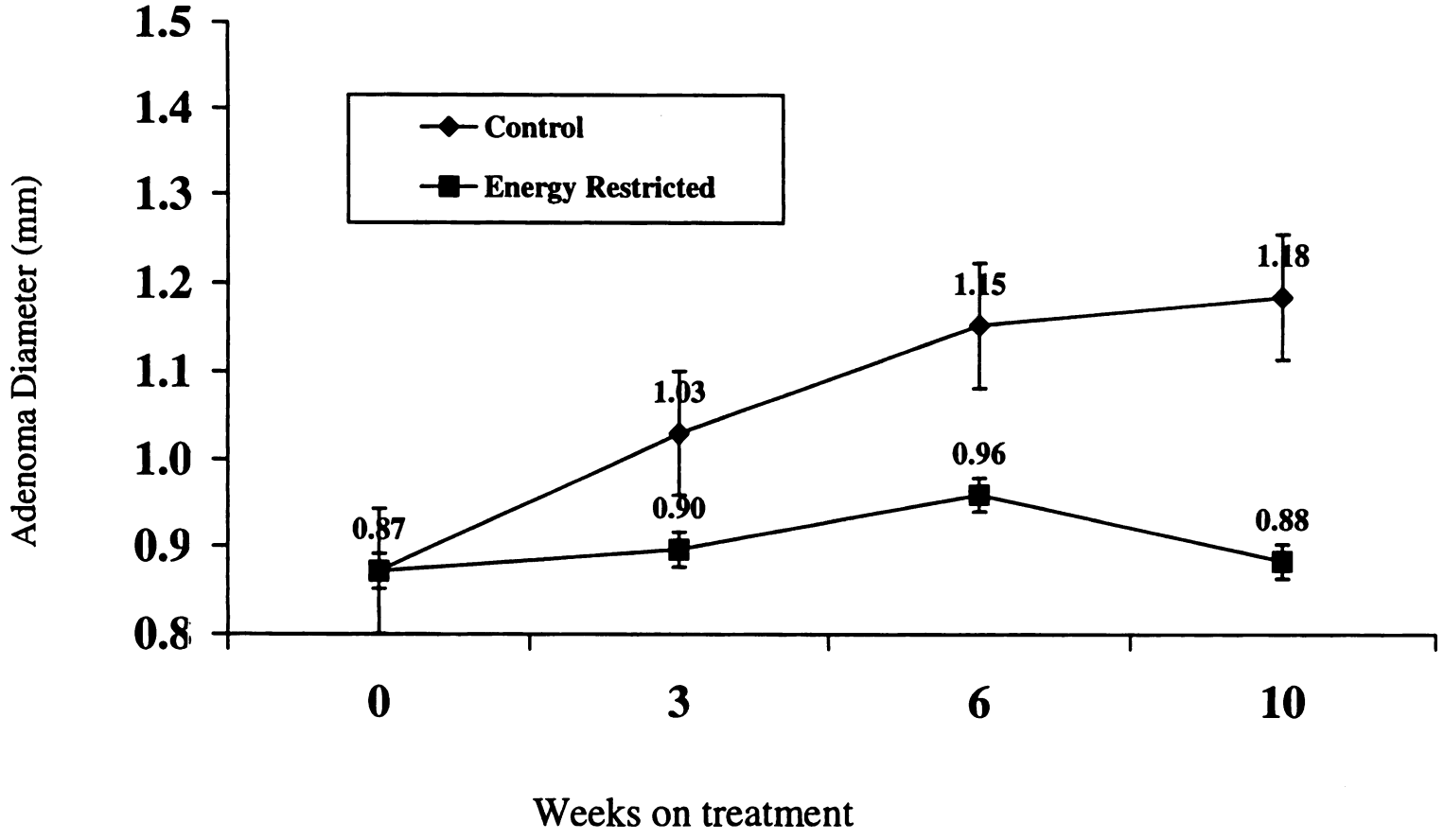

Figure 3. Small intestine adenoma diameters in *ad libitum*-fed control mice and 30% energy restricted mice at 0, 3, 6 and 10 weeks of treatment. The bars represent SEM. The diameter of the adenomas in the *ad libitum*-fed control group were significantly greater than the diameter of adenomas in the energy restricted group ($P<0.05$). There was no significant time effect ($P<0.05$).

Colon Tumor Number

The numbers of colon tumors at 3, 6 and 10 weeks of treatment were not significantly different (P=0.71) between the control and energy restricted groups (Figure 4). As there was no overall treatment effect, comparisons at the different time points were not conducted.

Colon Tumor Volume

Tumor volume in colons of the *ad libitum*-fed mice appeared to rise sharply after the third week of treatment and remained high for the remainder of the experiment (Figure 5). Colon tumor volume for the energy restricted group, however, remained steady throughout the experiment. The differences between the control and the energy restricted group were not significant (P<0.07).

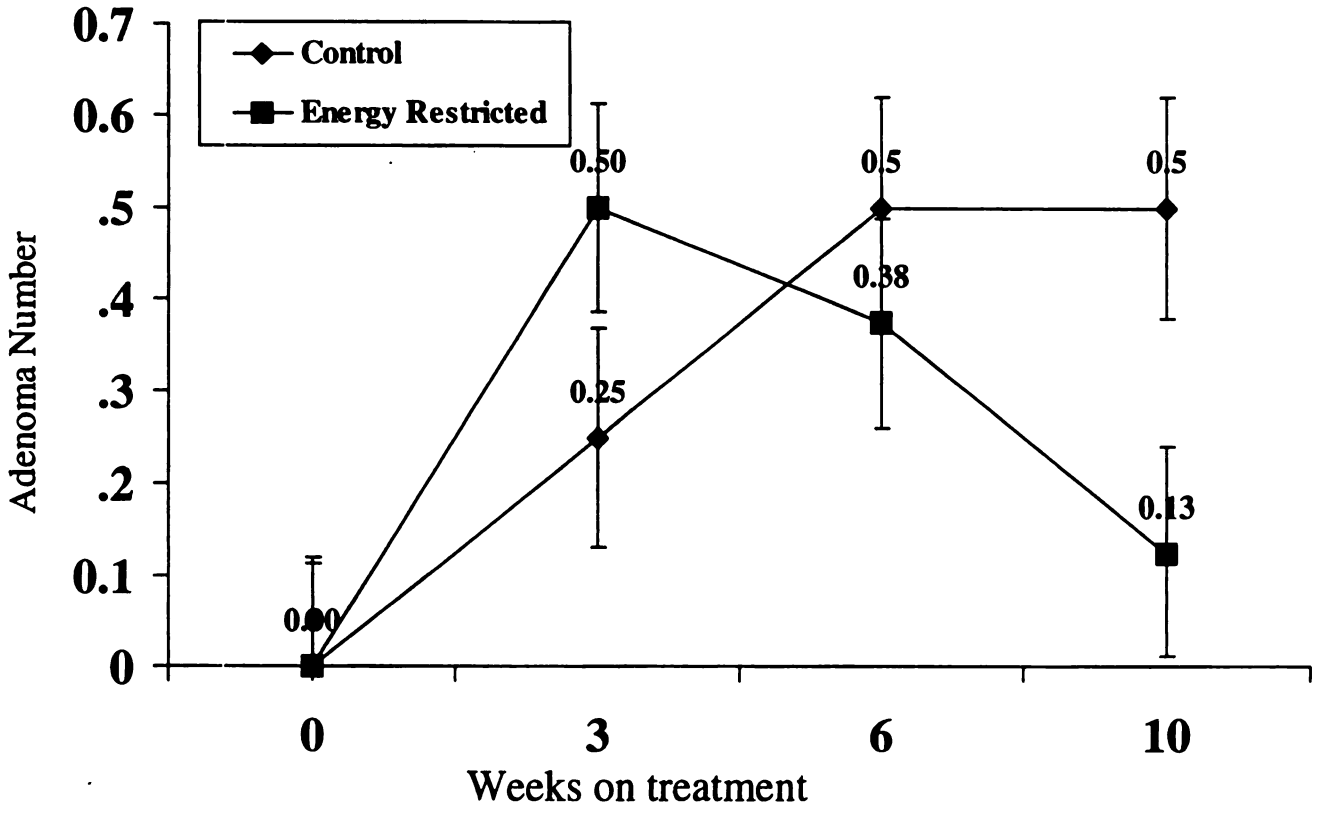

Figure 4. Tumor multiplicity (number of colon tumors/ number of mice) in *ad libitum*-fed control mice and 30% energy restricted mice at 0, 3, 6 and 10 weeks of treatment. The bars represent SEM. There was no significant treatment or time effect for colon tumor multiplicity.

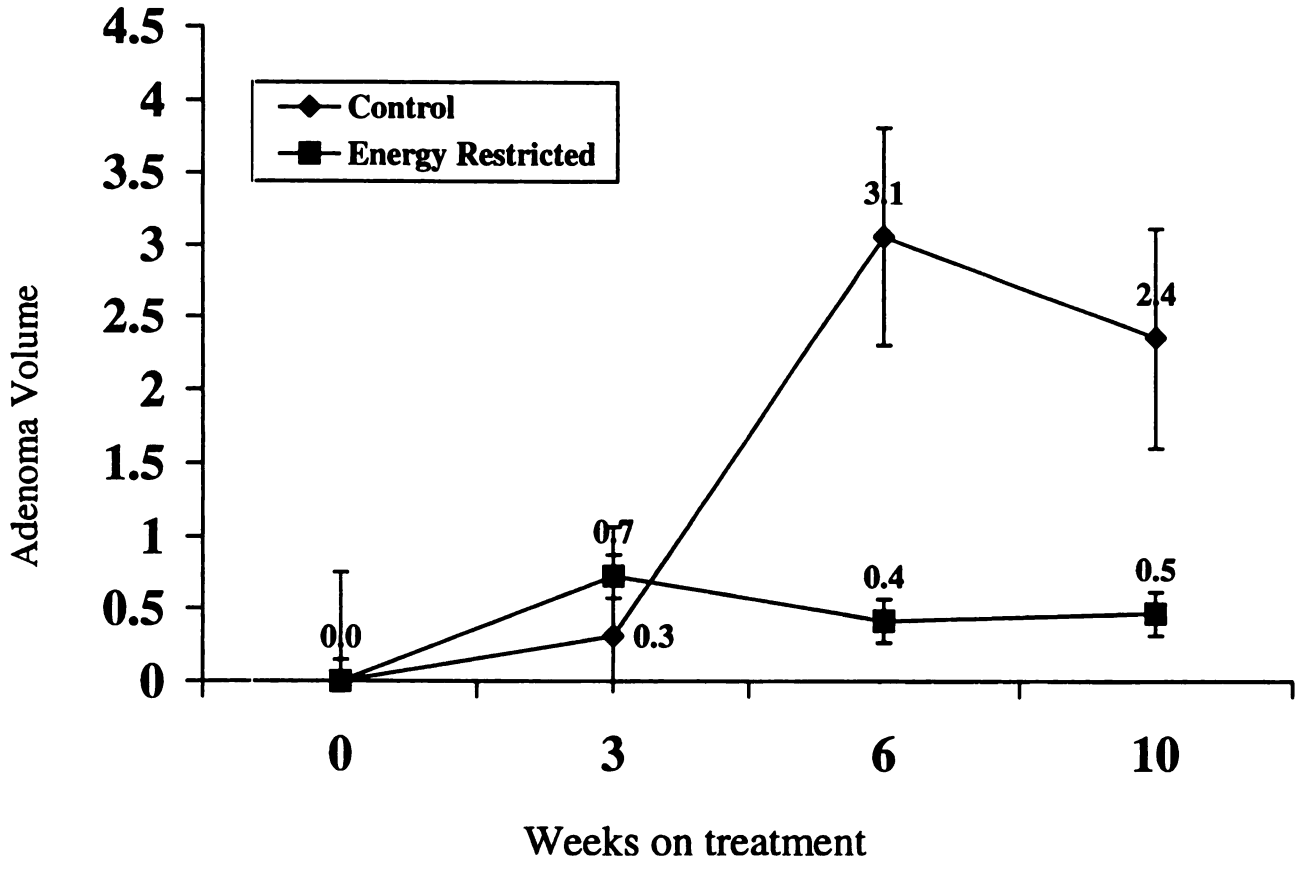

Figure 5. Colon tumor volumes for *ad libitum*-fed control mice and 30% energy restricted mice at 0, 3, 6 and 10 weeks of treatment. The bars represent SEM. There was no significant treatment ($P<0.07$) or time effect for colon tumor volume.

Gene Expression

A comparison of mRNA levels in tumor tissue from the small intestine of energy restricted animals and control *ad libitum*-fed Min mice revealed that 30% energy restriction is associated with changes in mRNA levels. Out of 1,200 genes studied, 11 genes (0.9%) in tumors from energy restricted showed a greater than 1.6-fold increase in expression compared to tumor tissue from ad libitum fed control mice. Over-expressed genes and their functional categories are listed in Table 2. The over-expressed genes include genes associated with immune function, growth factors and transcription factor, and genes related to apoptotic pathways.

Thirteen out of 1,200 genes (1%) showed a greater than 1.7-fold decrease in expression in tumors from energy restricted mice compared to tumors from control mice. The genes that were expressed at a lower level in the energy restricted mice and their functional categories are listed in table 3. Among the genes that are underexpressed are 4 proto-oncogenes or oncogene related proteins and one DNA excision repair gene.

Table 2. Over-expressed genes in tumor tissue of energy restricted mice[1].

Gene	*Fold ↑ Expression*	*Functional Category*
disabled homolog 2 (Drosophila)	4.3	Negative growth regulator
DIPEPTIDYL-PEPTIDASE I PRECURSOR (DPP-I) (CATHEPSIN C) (CATHEPSIN J) (DIPEPTIDYL TRANSFERASE)	3.5	Lysosomal protease
fibroblast growth factor 15 (FGF15)	3.3	Regulator of cell division
cytoplasmid dynein light chain 1; protein inhibitor of neuronal nitric oxide synthase (mPIN)	2.7	Neuronal protective protein
basic fibroblast growth factor receptor 1 precursor (BFGF-R; FGFR1); MFR; FLG	2.7	Role in wound healing
gamma interferon-induced monokine precursor (MIG); M119	2.3	Immune Function
CD14 monocyte differentiation antigen precursor; LPS receptor (LPSR); myeloid cell-specific leucine-rich glycoprotein	2.2	Immune Function
NF-kappa-B transcription factor p65 subunit (NF-kB p65); relA; NFKB3	1.6	Immune Function

[1]Expression of 1,200 genes was analyzed using cDNA arrays in tumor tissues obtained from *ad libitum*-fed and energy restricted Min mice. Fold increase in expression was obtained by taking the ratio of normalized levels of RNA corresponding to each gene in *ad-libitum* fed (control) and energy restricted animals.

Table 3. Under-expressed genes in tumor tissue of energy restricted mice[1].

Gene	*Fold ↓ Expression*	*Functional Category*
junB	3.5	Oncogene
L-myc proto-oncogene protein	2.8	Oncogene
transcription factor AP-1; c-jun proto-oncogene; AH119	2.6	Oncogene
xeroderma pigmentosum group G complementing protein (XPG); DNA excision repair protein ERCC5	2	DNA repair
proliferating cell nuclear antigen (PCNA); cyclin	2	DNA synthesis
monotype chemoattractant protein 3	2	Immune-related protein
Pleiotrophin Precursor (Ptn) (Heparin-Binding Growth-Associated Molecule) (HB-GAM) (Heparin-Binding Growth Factor 8) (HBGF-8) (Osteoblast Specific Factor 1) (OSF-1) (Heparin-Binding Neutrophic Factor) (HBNF).	2	Growth factor/ differentiation factor precursor
neurogenic differentiation factor 1 (NEUROD1)	2	Neuronal development and differentiation factor
thrombospondin 3 precursor (THBS3; TSP3)	2	Structural glycoprotein
endothelin b receptor (Ednrb)	2	Elevated in several carcinomas
related to Drosophila groucho gene (GRG); amino enhancer of split protein (AES); enhancer of split protein 1 (ESP1)	1.8	Involved in neurogenic cell differentiation
paired box protein 6 (PAX6); SEY	1.7	Involved in neuronal migration during development, mutated in congenital eye disorder, aniridia
wingless-related MMTV integration site 10b protein precursor (WNT10B); WNT12	1.7	Oncogene

[1]A decrease in expression of these genes was found as a result of energy restriction of the animals. Fold decrease in expression was obtained by taking the ratio of normalized levels of RNA corresponding to each gene in energy restricted animals and *ad libitum*-fed (control) animals.

DISCUSSION

The majority of adenomas in Min mouse occur in the small intestine, therefore, the anti-neoplastic effects of energy restriction were more apparent in this part of the intestine. In our experiment, the number of small intestine adenomas peaked at approximately 9-12 weeks of age in the *ad libitum*-fed control mice. Kang et al (1999) reported an increase in tumor number with age until about 12 weeks after birth. Adenoma diameter, however, increased linearly until the termination of the experiment (age 13-15 weeks). The slight drop in weight of the *ad libitum*-fed mice beginning at week 8 of treatment was likely due to intestinal bleeding from the large number of adenomas.

Weight gain in the energy restricted mice was nominal. Small intestine adenoma number increased during the first two weeks of energy restriction (32 adenomas per mouse) with no further increase with time of treatment (29 adenomas per mouse). The difference in number of small intestine adenoma between the *ad libitum*-fed and energy restricted groups increased with length of treatment. At 6 weeks of treatment there were 29% fewer adenomas in energy restricted mice and at 10 weeks of treatment there were 38% fewer adenomas in energy restricted mice. We have observed a similar degree of inhibition (36% decrease) of adenoma formation in the Min mouse with a 20% energy restriction in an earlier study (Mridvika et al., submitted).

Anti-carcinogenic effects of energy restriction have been well documented in spontaneous tumors and azoxymethane-induced colon tumors (Reddy et al., 1987). Several mechanisms have been postulated to explain the anti-cancer effects of energy restriction. Some of the proposed mechanisms include increased capacity for DNA

repair, decreased metabolic rate, decrease in oxidative stress, and changes in gene expression. Zhu *et al.* (1999) reported that energy restriction, in a dose dependent manner, up-regulated p27/kip1, a protein associated with cell cycle growth arrest, and down-regulated cyclin D1, a protein that promotes the progression of cells through the cell cycle. Changes in gene expression by energy restriction have also been reported by Lee *et al.* (1999). In the 6,000+ genes studied, 1.8% of the genes either increased or decreased expression as a function of aging in mouse gastrocnemious mucsle (Lee et al., 1999). Upon 24% energy restriction, a large number of these changes were completely reversed and a significant number were partially reversed. The expression of a similar number of genes (1.9%) was altered in intestinal tumors in our study.

The progression of normal-appearing intestinal epithelium to adenomas most likely resulted from molecular changes that facilitate the progression of tumorigenesis. These changes in the Min mouse are beginning to be elucidated including the loss of wild-type allele of the *Apc* gene, the possible increased expression of additional oncogenes, and/or decreased expression of tumor suppressor genes. Inhibition of tumorigenesis in the small intestine by energy restriction was accompanied by decreased expression of 4 oncogenic proteins. Jun B and c-jun proto-oncogene (transcription factor AP-1; AH119) are immediate early genes that are induced during G0/G1 (Bravo, 1999). These genes encode for molecules that can regulate the expression of other genes whose products are essential for progression through the G1 phase of the cell cycle. L-myc is a proto-oncogene protein from the myc family of oncogenes. Wnt10b overexpression has led to formation of mammary tumors in mice (Lane et al., 1997) and behaves like a proto-oncogene leading to formation of tumors in transgenic mice.

Energy restriction slows metabolism, which reduces the production of potentially toxic metabolic by-products of, which could cause macromolecular damage. The two-fold decrease in the expression of XPG DNA repair protein suggests a reduction in the need to repair damaged DNA. A similar argument has been offered by Lee et al. (1999), for the down regulation of repair enzymes in energy restricted mice in their aging experiments. PCNA, an auxillary protein for one of the DNA polymerase enzymes (Bravo, 1987), is also down-regulated by two fold. This down-regulation of PCNA is consistent with a slower metabolic rate and a smaller adenoma size observed in the energy restricted group.

A gene that exhibited the greatest increase in expression as a result of energy restriction was disabled homolog 2 (Dab 2), a mammalian structural homolog of Drosophila disabled (Dab). Disabled homolog 2 is a mitogen-responsive protein that is thought to be a negative regulator of growth as its expression is lost in ovarian cancer (Xu et al., 1998). Dab 2 may also regulate *Ras* pathways by competing with other molecules that bind Ras. Dipeptidyl-peptidase 1 (DPP I) precursor was also up-regulated in energy restricted animals. DPP I, a lysosomal cysteine proteinase is important in the intracellular degradation of proteins and is a central coordinator for activation of many serine proteinases in immune/inflammatory cells (Rao etal., 1997).

It is noteworthy that 50% of the genes that were up-regulated as a consequence of energy restriction are related to immune function. Basic fibroblast growth factor receptor 1 precursor (BFGF-R; FGFR1) is a potent mitogenic and chemotactic factor for endothelial cells and fibroblasts (Akimoto *et al.*, 1999) and may play an important role in scar healing. The gamma interferon-induced monokine precursor (MIG) is a member of

the platelet factor 4-interleukin-8 cytokine family that is expressed in murine macrophages specifically in response to IFN-gamma. Wong *et al.* (1994) also reported that MIG is overexpressed in energy restricted animals. CD14 monocyte differentiation antigen precursor; LPS receptor (LPSR) is responsible for the activation of B cells and hence forms an integral part of immune function. NF-kappa-B transcription factor p65 subunit (NF-kB p65) is yet another immune molecule up-regulated by energy restriction.

An immune modulatory effect of energy restriction has been reported. Most studies have suggested an impaired function resulting from weight loss induced by energy restriction (Nieman *et al.*, 1998; Shi *et al.*, 1997). However, others have suggested an immunostimulatory effect of energy restriction (Lim *et al.*, 2000). The parameters of immune function studied in these cases are probably responsible for the conflicting results.

Energy restriction in Min mice for a period of 10 weeks changed expression of genes in ways that slow tumor growth. An overall pattern emerging from this experiment was a decreased expression of oncogenes and growth factors, an increase in the expression of genes that slow down cell division, and a marked increase in genes involved in immune function. These results are consistent with previous studies that suggest that energy restriction reduces the need for DNA repair.

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