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ADIPOCITY RELATED PROTECTION OF INTESTINAL TUMORIGENESIS:
INTERACTION WITH DIETARY CALCIUM

A Dissertation
Presented for the
Doctor of Philosophy
Degree
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Shengli Ding
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DEDICATION

This dissertation is dedicated to my mother, ShuYing Ding
and to the memory of my father, GuangChang Yu
and my sisters, YuanYue Yu, XiaoHong Yu, LiJia Yu.

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ABSTRACT

Excess adipose tissue is a risk factor for developing colorectal cancer. However, the present studies demonstrate that lack of adipose-derived factor(s), such as adiponectin, due to the substantial loss of body fat on high dairy calcium diet could increase susceptibility to intestinal tumorigenesis. These studies suggest that a minimum amount or threshold level of adipose tissue may be required to significantly attenuate tumorigenesis.

In *Apc*^{Min/+} mice, consumption of high dairy calcium diet exhibited markedly reduced adipose tissue and increased tumor number. Our results showed that the high calcium diet reduced fat pad mass by 65%-82% in *Apc*^{Min/+} ($p<0.03$) in comparison with low calcium diet. *Apc*^{Min/+} mice on the high calcium diet exhibited an increase in tumor number (76 vs. 29, $p=0.009$). Moreover, β -catenin gene and cyclin D1 gene expression were significantly induced in intestinal tumor tissue of *Apc*^{Min/+} mice on high calcium diet. These effects were not directly resulted from high dietary calcium feeding, but rather associated with loss of body fat mass. Tumor load was not affected by the calcium diet on obese *A^y/Apc*^{Min/+} mice despite the fact that high dairy calcium diet produced a substantial loss of body fat, as there was still substantial residual adipose tissue remaining. Our results indicate that protective effects of calcium against colorectal cancer may be least apparent among lean individuals, suggesting that future studies of calcium and colorectal cancer should consider stratification of subjects according to adiposity.

In an *in vitro* environment to determine the adipose-derived factor(s) responsible, we utilized a co-culture system to observe the influence of human adipocytes on the growth of Caco-2, a human colon cancer cell line. We found that human adipocytes substantially suppressed proliferation of Caco-2 by 62.8%. In addition, we reported that human adipocyte conditioned medium inhibited growth of Caco-2 cells by 28.0%-65.6% compare to DMEM. These findings suggest the protective effect of adipocytes on colonic tumorigenesis. To further investigate if adiponectin, a protein hormone secreted from mature adipocytes, is responsible for this inhibitory effect, anti-human adiponectin-neutralizing antibody was added into the human adipocyte conditioned medium (HACM) and to the co-culture system. The antibody blocked the growth-inhibiting effects in both human adipocytes (HA) and HACM. Consistent with this, siRNA-mediated decrease in adiponectin protein in human adipocytes prevented the inhibitory effect of human adipocytes on Caco-2 proliferation. These data demonstrated that human adipocytes inhibit Caco-2 proliferation and that adiponectin is responsible for this effect.

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PART 1
INTRODUCTION

INTRODUCTION

Colorectal cancer is the 3rd most common cancer worldwide. The American Cancer Society estimates that the new cases of colon cancer will reach 112,340 and the new cases of rectal cancer will be 41,420 in 2007 in the United States (1). Diet appears to be the most important exogenous factor identified in the etiology of colon cancer. It has been estimated that 70% of colorectal cancer could be prevented by nutritional intervention (2).

The association between calcium intake and the risk of colorectal cancer was first investigated by Garland in 1985 (3). After a 19-year prospective study of about 2000 people in Chicago, they found a strong inverse correlation between colorectal cancer risk and dietary vitamin D and calcium intake. After that, several observational studies, and interventional studies were conducted. Mounting evidence demonstrates that dietary calcium is inversely associated with the risk of colorectal cancer (4-9). Both in vitro and in vivo studies suggested that high calcium intake may reduce cytotoxicity of fecal water (10, 11) or inhibit epithelial cell proliferation (12, 13). However, our preliminary observations suggested that high calcium intake paradoxically promoted intestinal tumor growth in *Apc*^{Min/+} mice, and this effect was accompanied by a profound loss of adipose tissue. Since we have previously demonstrated that increasing dietary calcium effectively reduces adiposity (14, 15), we hypothesized that this excessive loss of adipose tissue resulted in increased tumor susceptibility in *Apc*^{Min/+} mice on the high calcium diet.

Adipose tissue is an active endocrine and metabolic organ that can have effects on the energy balance and lipid metabolism of other tissues. It also regulates their physiology through the release of hormones such as leptin, adiponectin, resistin and tumor necrosis factor- α (TNF- α). The relationship between excess body weight and the incidence of colon cancer has been well established in epidemiological studies (16-20). Obesity had been consistently associated with higher risk of colorectal cancer in men (relative risks of approximately 1.5-2.0) and women (relative risks of proximately 1.2-1.5) in both case-control and cohort studies (21). Adiponectin, also called adipocyte complement-related protein (Acpr30), is a cytokine secreted from mature adipocyte. Adiponectin acts as an insulin-sensitizing hormone (22, 23) but also has anti-inflammatory (24) and anti-atherogenic effects (25, 26). Unlike leptin and TNF- α , circulating adiponectin levels are decreased with increasing adiposity (27, 28). Low levels of adiponectin may thus provide a link between obesity and risk of colorectal cancer.

Consequently, in our first study, we investigated whether the increased tumor susceptibility of *Apc*^{Min/+} mice on high calcium diet was due to the substantial loss of adipose tissue. Secondly, we designed experiments to evaluate the inhibitory effect of human adipocytes on Caco-2 cell proliferation *in vitro*. We also identified that adiponectin is at least in part responsible for this inhibitory effect.

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PART 2
LITERATURE REVIEW

1. Colorectal cancer: general aspects

1.1 Prevalence of colorectal cancer

Colorectal cancer is the 3rd most common cancer worldwide and the second most common cause of cancer death in men and women, with an estimated 112,340 new cases of colon cancer and 41,420 new cases of rectal cancer in 2007 in the United States. Combined, they will cause about 52,180 deaths (1). Men and women are affected almost equally, with over 1 million cases annually diagnosed worldwide (2). Typically the rates of colon cancer incidence tend to be higher in developed countries. The highest rates of incidence are in North America, Australia, and New Zealand, whereas the lowest rates are in Africa, Asia and Latin America (3). Schottenfeld's study showed that the risk of colon cancer increases in migrants after a period of 20-30 years following migration to a high-risk country, and that the risk of developing colon cancer decreased after migrating from a high-risk area to a low-risk country (4).

1.2 Risk factors

1.2.1 Non-dietary factors

Age is a risk factor for the development of colorectal cancer, with 90% of colorectal cancer patients being over the age of fifty; however, risk is also increase in younger individuals who had a previous adenomatous polyp, or a history of clinical bowel inflammation (3). According to European Network of cancer Registries, in Europe, about 70% of patients with colon cancer are over 65

years of age. Colon cancer is rare under the age of 45. In the age group 45-54 colon cancer incidence is about 20 per 100,000 per year and thereafter increases at a much higher rates (55 per 100,000 per year for aged 55-64, 120 for aged 65-74 and 200 per 100,000 per year for those older than 75 years of age) (5).

Smoking has been consistently associated with adenomatous polyps. In the United States, about one in five colorectal cancer cases in men and about one in eight colorectal cancer cases in women may be due to smoking (6). Smoking seems to influence the early stages of the process, when normal cells become cancerous. One study has suggested that smoking early in life is likely to increase risk of colon cancer, and approximately 20% of the colon cancers in men appear to be attributable to smoking (7). Recent studies show that smokers are 30% to 40% more likely than nonsmokers to die of colorectal cancer.

Many research studies have found people who have a high body mass index (BMI) are at an increased risk of colorectal cancer. Any additional weight above the healthy range appears to increase the risk of colorectal cancer. Some evidence indicated that a tendency to store fat in the abdominal area may also increase the risk of colorectal cancer. These will be discussed in detail below.

Race may be another risk factor for developing colorectal cancer. For example, colorectal cancer rates are lowest for Hispanics, while African Americans, both men and women, have the highest incidence and mortality rate. The five-year survival rate is much lower among African Americans than Whites (2). The reason for this racial difference is unclear and need to be studied further to better establish the underlining causes.

1.2.2 Dietary factors

Diet appears to be the most important exogenous factor identified in the etiology of colon cancer. It has been estimated that 70% of colorectal cancer could be prevented by nutritional intervention (8). Case-control studies have identified both promoting and protective factors. Generally, high vegetable, low fat diets have been associated with a lower risk of colorectal cancer.

Vegetables and fruits contain a large array of substances, both micronutrients, such as carotenoids, folate and ascorbate; and phytochemicals, such as phenols, flavonoids, isothiocyanates, and indoles. Vegetables are also rich in fiber. Early evidence indicated that diets rich in vegetables and fruits substantially protect against colon cancer. In a recent review by Steinmetz and Potter (9), 15 of the 21 case-control studies examined showed statistically significant inverse associations between one or more vegetable/fruit categories and colon cancer risk. Four of the studies did not show significant inverse associations for any vegetable/fruit category, and in two studies statistical significance was not reported. Steinmetz and Potter also concluded that the protective effect of vegetables seemed to be most clear for raw vegetables vs. cooked and the green vs. yellow/orange vegetables. However, recent intervention and prospective studies of colorectal adenoma recurrence and colorectal cancer have not found protective associations, resulting in skepticism regarding the independent role of plant foods in cancer prevention (10-13). For instance, in the Netherlands Cohort Study on Diet and Cancer (12), the authors use a validated 150-item food frequency questionnaire.

After 6.3 years of follow-up (1986–1992), over 1,000 incident cases of colorectal cancer were registered. Using case-cohort analysis, the authors concluded that for colon cancer, no statistically significant associations with total vegetable intake or total fruit intake were found. However, among women, an inverse association was observed with vegetables and fruits combined (for the highest quintile vs. the lowest, the rate ratio (RR) was 0.66 (95% confidence interval (CI): 0.44, 1.01)). For rectal cancer, no statistically significant associations were found for vegetable consumption or fruit consumption or for specific groups of vegetables and fruits; only *Brassica* vegetables showed an inverse association in women. Another example is a large, prospective study in older Americans (13), the authors concluded that neither whole grain, fruits and vegetables, nor fiber consumption was independently associated with risk of incident colon cancer. Among men, consumption of vegetables, or a great variety of vegetables, was associated with a marginally lower risk of developing colon cancer, though this was not significant.

The term “dietary fiber” encompasses a complex mix of mostly non-digestible plant materials with variable effects on gastrointestinal physiology (14). The concept that a diet high in fiber, especially from fruits and vegetables, lowers risk of colorectal cancer has been around for more than four decades, starting from the observation that certain groups of people in Africa who eat a high-fiber diet also have low rates of colorectal cancer (15). Since this original idea, the relationship between fiber, fruits and vegetables in general, and colorectal cancer risk has been evaluated, with somewhat conflicting results. Some studies suggest that fiber can reduce risk, while others have found no effect. For example, the result from a

large cohort European study on diet support role of fiber as a protective factor for colon cancer (16). A large female cohort from the Women's Health Study indicated that high intake of legume fiber was associated with a lower risk of colorectal cancer (RR=0.60, 95%CI: 0.40, 0.91, $p=0.02$) (17). The beneficial effect of dietary fiber was suggested to be a consequence of the ability of fiber to bind and reduce the concentration of bile acids in fecal water (18). Nevertheless, recent epidemiological data tended not to support this influence. One recent prospective study of fiber and colon cancer risk (13) reported that high intake of fiber was not related to lower risk of colon cancer, and this null finding is consistent with several other prospective studies (19-22).

The relationship between alcohol intake and colorectal cancer risk has been controversial, but the majority evidence now indicates that consuming large amounts of alcohol increases the risk of colorectal cancer (23). More recently, investigators have addressed this hypothesis using data from the Pooling Project of Prospective Studies, which combined data from eight large prospective studies (24). In the pooled analysis, compared to nondrinkers of alcohol, individuals who consumed 30 to 45 g/d of alcohol had a multivariate relative risk of 1.16 (95% CI: 0.99, 1.37) and those who consumed 45 g/d of alcohol had a multivariate relative risk of 1.42 (95% CI: 1.16, 1.73). Alcohol was not related to risk among those who took multivitamins, most of which contain folic acid. In general, risk of both proximal and distal colon cancers and rectal cancer appear to be increased by high alcohol intake, although the reason for this increased risk is not entirely clear.

Meat consumption has been associated with colorectal neoplasia in the

epidemiological literature, but the strength of the association and types of meat involved have not been consistent. World Cancer Research Fund (WCRF) indicated that red meat “probably” and processed meat “possibly” increases risk of colon cancer (23). Case-control studies of colorectal cancer conducted in the United States and European have generally reported the increased risk of colon cancer associated with red or processed meat (25, 26). Results from another prospective study of American women showed no evidence of an association between meat and colorectal cancer (27), which is consistent with the conclusion made from previous studies (25). More recently, results of the Cancer Prevention Study II Nutrition Cohort (28) showed that prolonged high consumption of red and processed meat might be associated with an increased risk of cancer of the distal portion of the large intestine; however, the increase was not statistically significant. The mechanisms underlying the association between colorectal cancer risk and high intake of red and processed meat are uncertain. Controlled human intervention studies have raised the possibility that the endogenous nitrosation that arises from ingestion of heme iron may account for the increased risk associated with red and processed meat consumption (29, 30).

2. Dietary calcium and colorectal cancer

The adequate intake (AI) for calcium in adults of 19-50 y and 50-70 y are 1,000 and 1,200 mg/day respectively. However, many people in the United States do not consume recommended levels of dietary calcium. According to the National Health and Nutrition Examination Survey from 1999-2000 (NHANES), median calcium intake for men ages from 19 to 50 years was approximately 900 mg/day, for women was about 800 mg/day, which is approximately 70% of the AI for that age group (31). Continuing Survey of Food Intakes by Individuals 1994-1996, 1998 (CSFII 1994-1996, 1998) and NHANES 1999-2000 data both show that African Americans in all age groups have lower mean intakes of calcium than non-African Americans in both men and women (31). Low calcium intake is related to various chronic diseases. In addition to skeletal disorders, calcium deficit increases the risk of malignancies, particularly of colon, breast and prostate gland, of chronic inflammatory and autoimmune diseases, as well as of metabolic disorders (metabolic syndrome, hypertension) (32).

2.1 Epidemiology

Calcium and calcium-containing foods have been evaluated in numerous studies of colon cancer. Ecological studies are the first epidemiological evidence to examine the associations between colorectal cancer rates occurred in different populations and their corresponding dietary habits. When the data for milk or milk product consumption is plotted against colorectal cancer mortality, it

emerges that *per capita* consumption of milk or milk products is inversely associated with mortality of colorectal cancer (33). Sorenson *et al* (34) have noted that the areas in rural Finland, where calcium consumption is high, have one of the lowest colon cancer incidence rates among the developed countries, whereas Helsinki has a higher incidence rate and a lower level of calcium intake. Traditionally, ecological studies are useful, but because they do not consider other factor, such as environmental and lifestyle variations, which limit conclusions regarding the relationships between milk or calcium consumption and colorectal cancer.

The analytical observational epidemiological literature on the association of calcium and colon cancer is somewhat inconsistent, but inverse associations have more frequently been found (35-45). The first long-term prospective study of the association of dietary calcium and vitamin D and the risk of colon cancer was reported by Garland and coworkers (46). After a 19-year prospective study of 1965 men in Chicago, they found a strong inverse correlation between colorectal cancer risk and dietary vitamin D and calcium intake. Early prospective effect of calcium was also described from case-control studies in the Southwest United States (47) and in Australia (48). In 1988, Sorenson *et al* summarized all of these studies and emphasized the inverse relationship with calcium consumption in age-adjusted colon cancer incidence rates (49). Recent epidemiologic evidence, including 7 cohort studies of colorectal cancer (35-37, 39-41, 45) and 3 case-control studies (38, 42-43), generally reported inverse associations between calcium intake and overall risk of colorectal tumors, and suggested a 30-40%

reduction in risk at high levels of intake (35-37). Cho *et al* (44) pooled 10 prospective studies in a meta-analysis and reported that calcium intake was inversely related to the risk of colorectal cancer. The relative risk (RR) for the highest versus the lowest quintile of intake was 0.86 (95% CI: 0.78, 0.95; $P_{\text{trend}} < 0.02$) for dietary calcium and 0.78 (95% CI 0.69 to 0.88; $P_{\text{trend}} < 0.001$) for total calcium (combining dietary and supplemental sources).

In the United States, colon cancer cases in the left-sided or distal colon constitute about two-thirds of all colon cancer cases (54). A protective effect of higher dietary calcium intake against colon cancer has been reported for more distal colon tumors than proximal and rectum tumors (36, 44, 50, 51-53). In the cohort study of women done by Terry *et al* (36), calcium was inversely associated with distal colon RR=0.45, 95% CI: 0.26, 0.79) and rectal cancer (RR=0.70, 95% CI: 0.45, 1.09). In Peters' study (50) the protective associations were strongest for tumors of the distal colon, with no association between calcium and rectal tumors. In contrast, in the Cancer Prevention Study II Nutrition Cohort (37), a high total calcium intake was related to a reduced risk of proximal colon cancer, but not of distal colon and rectal cancer. In other epidemiologic studies, associations between calcium intake and rectal tumors were equivocal, with some studies showing significant protective associations (43, 55), and with others showing statistically non-significant protective associations (56, 57).

Although a protective effect of dietary calcium is suggested by many studies, such a relationship is not found in all studies. Observational epidemiologic studies published through the mid-1990s provided no strong support for a protective

association between calcium intake and colorectal cancer risk (58, 59). A recent cohort study conducted in women also reported no association (60). Studies that showed no significant inverse association with calcium were generally limited by a relatively small sample size and thus were underpowered to detect risk reductions. In addition, inaccurate recall of dietary and supplemental intake might not reflect the usual intake during the observation period, resulting in an attenuated calcium-colorectal cancer association.

Thus, although there is animal and epidemiological support for the hypothesis that higher calcium consumption may contribute to a modest reduction in the risk of colon cancer, clinical trials will be needed to ultimately fully address the hypothesis.

2.2 Intervention trials

It is extremely difficult to conduct randomized controlled trials of sufficient size and duration to isolate the protective effect of any nutritional supplement (61); consequently, it is generally necessary to rely on biomarkers and intermediate end points. Two major different end points have been used as intermediate biomarkers of risk in colorectal neoplasia. An early intermediate biomarker is colorectal epithelial cell proliferation. A late intermediate biomarker of risk is the formation of adenomatous polyps, which usually have been investigated in volunteers who have previously had adenomatous polyps and therefore are at risk for recurrence.

2.2.1 Human trials of calcium and colorectal epithelial cell proliferation

Incubation techniques and methods that measure endogenous markers with DNA synthesis have been used to determine proliferating cells in the laboratory (62). The most commonly used methods of measuring colorectal epithelial cell proliferation are the H^3 -thymidine method (63), the bromodeoxyuridine (BrdU) method (64), and the proliferating cell nuclear antigen (PCNA) method (65). H^3 -thymidine method is very sensitive and the disadvantages are dealing with radioactive material and time consuming. Alternatively, incubation with BrdU has been used. In the PCNA method, no incubation and incorporation of a labeling agent are required, but PCNA method has been criticized for being more difficult to count, because the method yields cells that are labeled to varying intensity.

Regardless of which method has been used in measuring proliferating cells, there is controversy regarding the effect of calcium supplements upon proliferation. The early studies of calcium administration showed statistically reduced labeling indices. Lipkin and Newmark reported the first human study of the effect of calcium administration upon the colon in 1985 (66). In that uncontrolled study, administration of 1,250 mg of elemental calcium to subjects with a familial history of sporadic adenomas resulted in pronounced changes in cell proliferation. In the following several years, a series of studies (67-69) supported that supplemental dietary calcium decreased hyperproliferation of colonic epithelial cells. Buset's research (67) on 9 patients at risk of developing colorectal cancer who were supplemented with 1,500 mg/d of calcium for 4-8

weeks concluded that this calcium intervention inhibited the proliferation of colonic epithelial cells. Moreover, each colonic mucosal biopsy exhibited a decrease in proliferation when they were cultured in vitro with a high level of CaCl_2 . Further evidence was obtained in a similar experiment done by Lipkin in 1989 (69).

In the past two decades, some studies confirmed that dietary calcium could decrease colonic epithelial cell proliferation (70-73). For instance, Bostick's study (72) was carried out on patients at increased risk of colon cancer and found that calcium supplementation decreased the size of the proliferative compartment in the colonic crypts. Holt et al (73) also designed a randomized, single-blinded trial to determine whether increasing dietary calcium via low fat dairy food altered colonic biomarkers toward normal, and reported that increasing calcium up to 1,200 mg/d in subjects at risk for colonic neoplasia reduces the epithelial cell proliferation activity. However, other studies could not confirm this effect (74-78). Van Gorkom (74) performed a randomized controlled trial in 86 patients (28 treated with placebo and 30 with calcium + placebo). Epithelial cell proliferation was determined by dividing the number of 5-bromo-2-deoxyuridine labeled nuclei by the total number of nuclei $\times 100$ (labeling index, LI). No difference was observed in total LI. They concluded that colonic epithelial cell proliferative activity throughout the colon of sporadic adenoma patients is not affected by supplementation with 1 g of calcium. In double-blinded, placebo-controlled randomized trials, neither Armitage *et al* (75) nor Alberts and coworkers (77) found a significant effect of calcium carbonate supplements on cellular

proliferation rates in colon mucosal biopsies. Therefore, Bostick (79) concluded that the combined results of all of the published studies of calcium supplementation and colorectal epithelial cell do not support the hypothesis that calcium supplementation reduces the rate of proliferation of the colon epithelium in humans.

Several reviews have discussed the reason for the inconsistency in the literature regarding the effects of oral calcium on colonic epithelial cell proliferation (79-81). Possible explanations include the uncontrolled nature of the early studies and the sample size as related to the clinical importance of the estimated treatment effect. Variations in sample size may cause the bias in the results. For example, Van Gorkom's trial (74) was conducted with 15 patients, and Buset's work included only 9 patients, whereas in some studies, the sample size was approximately 100 patients (78). Furthermore, there are many methodological problems that have been recognized. As mentioned in Bosick's review, the different trials took biopsies for cell proliferation measurements at different time points from one another. The studies also differed on use of bowel preps and the different studies use different laboratory methods for assessing cell proliferation. Thus, attempts are being made to improve the standardization of the population studied and the methods with which the assay end points are measured.

The conflicting evidence on the question whether dietary calcium does reduce colonic epithelial cell proliferation raises two key questions: (1) Is cell proliferation a good biomarker to examine the effect of dietary calcium on colorectal cancer risk, and (2) Is calcium supplementation a potential intervention

to reduce the risk of colorectal cancer?

2.2.2 The effect of dietary calcium on colorectal adenomatous polyps

The proliferative index does not appear to be a reliable predictive tool for future colorectal neoplasia, and Sandler (82) has suggested that adenomas serve as a better-proven and more reliable intermediate. Since almost all colorectal cancers develop from adenomas, and 5% of all adenomas develop into cancer (83), studies regarding the development of colorectal adenomatous polyps as an end point may be reasonable. Many clinical trials have been done to investigate the effect of dietary calcium on adenomatous polyps (84-91). Since it is difficult to conduct a randomized controlled trial large and long enough to observe the protective effect of dietary calcium, so far only two groups have succeeded in completing such trials. Baron described the first study of calcium prevention of adenomatous polyp recurrence in 1999 (84). They randomly assigned 930 subjects (mean age, 61 years; 72 % men) with a recent history of colorectal adenomas to receive either calcium carbonate (3 g (1,200 mg of elemental calcium) daily) or placebo, with follow-up colonoscopies one and four years after the qualifying examination. The adjusted risk ratio for any recurrence of adenoma with calcium as compared with placebo was 0.85 (95 % CI: 0.74, 0.98; $p=0.03$). The adjusted ratio of the average number of adenomas in the calcium group to that in the placebo group was 0.76 (95 % CI: 0.60, 0.96; $p=0.02$). Shortly after the first paper was published, a second randomized trial was done in 2000 (85), with 665 subjects with a previous history of colorectal adenoma; this study compared 3-arms: calcium 2,000 mg, fiber 3,500 mg and placebo group. The calcium group showed a significant

reduction for adenoma recurrence, odd ratio (OR)=0.66 (95% CI: 0.38, 1.17). Calcium supplementation for three years gave a modest reduction in the risk of adenoma recurrence. Similar to these two large clinical trials, other studies observed a decreasing risk of colorectal adenoma with an increasing intake of calcium (87-91).

2.3 Mechanisms behind the inhibitory effect of dietary calcium on colorectal cancer risk

2.3.1 Reduce the cytotoxicity of fecal water

The mechanism through which calcium may prevent colorectal cancer is not certain. One major hypothesis from Harold Newmark and coworkers (92) is that fatty acids and bile acids are the colonic components most responsible for inducing epithelial cells in the direction of carcinogenesis. They also emphasized that colonic fatty acids and bile acids would need to be in the aqueous phase of colonic contents in order to interact with the colonic mucosa and to stimulate pathogenic changes. They then postulated that if an excess of calcium entered the colon, then soluble bile acids and fatty acids would be precipitated and therefore no longer are able to alter the epithelium. This calcium precipitation of cytotoxic substances in feces hypothesis was confirmed in both human and animal studies. Van der Meer and coworkers (93) studied the fecal composition of 12 volunteers who fed either a regular diet or regular diet supplemented with 35 μ M of inorganic calcium per day in a crossover experiment. They demonstrated that the provision of supplemental calcium increased the amount of calcium appearing in

the stool when compared to volunteers not taking supplement calcium. The bile acid output increased by 35%, and there was a shift of the bile acid composition to an increased ratio of trihydroxy to dihydroxy bile acids. They then concluded that calcium would reduce damage to the colonic epithelium from fecal water. The same group did another experiment in rodents (94). They added low-fat dried milk powder to a Western-style diet in rodents and showed a similar reduction in the cytolytic activity of reconstituted fecal water. Later they fed volunteers with dairy calcium in dried powdered milk daily. The high milk calcium diet significantly decreased the cytotoxicity of fecal water from 68% to 28% (95). Calcium also has been shown to reduce the colonic content of diacylglycerol formed by bacteria, which may activate cellular transduction pathways and has been postulated as increasing proliferation in the colonic epithelium (96).

2.3.2 Inhibit epithelial cell proliferation

Another hypothesis is that calcium might directly influence proliferation by inducing cell differentiation. As cancer is predominantly a disease of disordered balance between proliferation, differentiation, and apoptosis, disruptions in the function of the calcium-sensing receptor (CaR) could contribute to the progression of neoplastic disease. (97). CaRs have been identified in human colon cancer cell lines (98, 99) and malignant mucosa of the large intestine (100). Furthermore, immunohistochemical studies of human colon carcinomas indicated that CaR protein was most highly expressed in well-differentiated regions of the tumor and nearly lacking in poorly differentiated regions (100, 101). These data

suggest that expression of functional CaR may contribute to the progression of colon carcinomas. The activation of CaR in colon epithelial cells is followed by the activation of protein kinase C (PKC). Early study by Weinstein (102) has shown that in human colonic mucosa total PKC activity was reduced compared to normal colonic tissues. Frey et al (103) have shown that PKC signaling is involved in a program of cell cycle withdrawal in intestinal epithelium associated with down-regulation of cyclin D1 and differential induction of the p21^{waf1/cip} and p27^{kip1} inhibitors of cyclin-dependent kinase (CDK) activity. Therefore, PKC activation through CaR provoked by dietary calcium may be critical for its antiproliferative, differentiation-inducing effects on human colon epithelial cells.

2.3.3 Target prostaglandin (PG) pathway

Cyclooxygenase-2 (COX-2) is an inducible enzyme that regulates prostaglandin synthesis and is over-expressed in animal models of colon carcinoma (104-106). Studies have elucidated the regulation of COX-2 expression and have identified prostaglandin E (PGE₂) receptors through which prostanoids exert their biological effects (105). COX-2-derived primary PG generated in colorectal tumors, PGE₂, is known to stimulate cell migration, proliferation and tumor-associated neovascularization while inhibiting cell death. A very recent study indicates that calcitriol inhibits the PG pathway in prostate cancer cells by decreasing COX-2 expression and PGE₂ receptors (107). Thus, calcitriol may be an attractive therapeutic option for the treatment of colorectal cancer.

2.4 Dairy food and colorectal cancer

Both case-control studies and prospective studies pointed out that milk is the individual dairy food with the strongest influence on colorectal cancer risk (108). Larsson et al (109) in their Cohort of Swedish men Trial found that colorectal cancer is influenced by total dairy food consumption (multivariate rate ratios (RRs) was 0.46). The effect of milk also reaches the significance (RR was 0.53.). A pooled analysis of ten cohort studies carried out by Cho et al (44) suggested that milk was inversely related to colorectal cancer. Compared with participants who consumed less than 70 g of milk per day, the relative risk (RR) for colorectal cancer was 0.85 (95% CI: 0.78, 0.94) for those who consumed 250 g/d or more. Other dairy foods (cheese and yogurt) have been analyzed, but there was no statistical significance with relation to colorectal cancer risk.

In addition to the calcium content in the dairy foods, other factors, such as conjugated linoleic acid, sphingolipids, butyric acid and milk proteins, have been linked to a reduction in colon cancer. Growing evidence indicate the impact of vitamin D status on the interaction between calcium intake, dairy intake and colon cancer risk. Animal studies showed that dietary calcium and vitamin D status were co-modulators of colon cancer and that vitamin D deficiency abolished the protective effects of calcium on tumor formation (110). Cho et al (44) presented data suggesting that the benefit of higher calcium intake may depend on high vitamin D intake. Given that vitamin D status might modulate effect of calcium on colon cancer risk, measurement of plasma levels of 1,25-(OH)₂ vitamin D and 25-(OH) vitamin D may generate a better testing of hypothesis on the roles of milk in colon cancer etiology.

3. Pathology and biology of colorectal cancer

3.1 ACF-Adenoma-Carcinoma sequence

The definition of an adenoma-carcinoma sequence in colorectal cancer (CRC) was first made by Basil Morson (111). This theory states that there is a progressive mutation of oncogenes and loss of the function of tumor suppressor genes (112), and the pattern of change that occurred in certain genes in colorectal cancers might be reflected in the different stages in development of tumors during the transition from normal mucosa to polyp and subsequent cancer (Fig.1). Thus, APC mutations occurred in early adenomas, while, mutations in the p53 gene occurred later in the sequence, although prior to metastasis. The mutated genes in CRC include those involved in the Wnt pathway, namely APC, and β -catenin, genes involved in apoptosis including p53 and the mismatch repair genes, human mutL homolog 1 (hMLH1), human mutS homolog 2 (hMSH2) and human mutS homolog 6 (hMSH6), cell cycle check point genes such as p14, p16 and genes for growth factors, their receptors and signaling (TGF β -receptor 2, Smad4, K-ras) (113).

The earliest identifiable lesion in colon cancer formation is the aberrant crypt focus (ACF). The crypts are larger or longer than normal crypts with no stratified and depolarized nuclei (114). The investigation of ACF by using 25 different genetic markers, such as microsatellite instabilities and mutations of APC, H-ras, K-ras, p53, deleted in colorectal carcinoma (DCC), and DNA repair genes hMLH1, hMSH2, showed no difference between hyperplastic ACF and normal mucosa (115). In dysplastic ACF, both crypts and cells have different degree of

abnormalities, with enlarged, elongated and sometimes stratified and depolarized nuclei. It has similar histopathological manifestations of adenomas (116). Dysplastic ACF does appear to harbor mutations in K-ras or APC. If a ras gene mutation occurred first, ACF would not be dysplasia, and if an APC mutation was the first to occur, such as familial adenomatous polyposis (FAP), it would result in a dysplastic ACF, whose progression was driven by subsequent K-ras and other gene mutations (117). Thus, alterations in APC, which result in over-activation of the Wnt signaling pathway (discussed below), appear to initiate tumor formation in the colon. Subsequent alterations in other genes play a role in tumor growth and tissue invasiveness.

3.2 Genetic basis in colorectal carcinogenesis

It is now accepted that colorectal cancer develops by genetic alterations. These alterations do not directly affect the cell biology of the tumor, but result in loss of genomic stability, which contributes to the accumulation of mutations in tumor

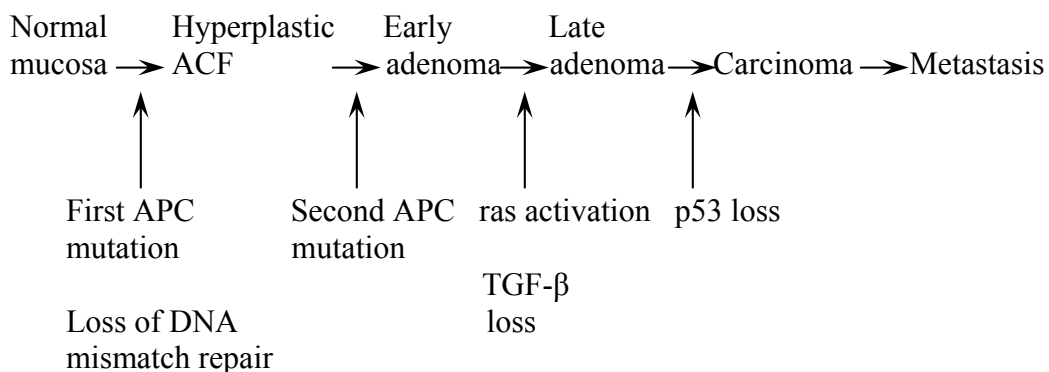


Figure.1 The multistep model for colorectal carcinogenesis

suppressor genes and oncogenes (118). There are two major pathways in colorectal carcinogenesis. One is the chromosomal instability pathway (CIN), and the other is a pathway involving microsatellite instability (MSI).

3.2.1 Chromosomal instability pathway

This pathway is characterized by allelic losses on chromosome 5q (APC), 7p (p53), and 18q (DCC/Smad4), and is therefore called the chromosomal instability (CIN) pathway (119). CIN pathway is a model represented by FAP, which is following ACF-adenoma-carcinoma sequence, that is, one germline mutation and one somatic mutation in the APC gene, followed by mutations of K-ras, and subsequently mutations of p53 and deletion on chromosome 18q.

APC gene

Mutations in the APC gene on human chromosome 5q21 locus are considered as one of the earliest events in the initiation and progression of colorectal cancer. Loss of APC caused by mutations in both alleles was found in a majority of FAP patients as well as in > 80% of sporadic CRCs and adenomas (120).

Many APC mutations have now been identified, both in the germ line in FAP patients, and somatically in sporadic CRCs. The classical mutations happened between codons 178 and 309 and between codons 409 and 1580, that is exons 5-8, 9-14 and the first half of exon 15 (121). In DNA sequence of APC, the most important functional domains of the APC gene appear to be the first serine alanine methionine proline (SAMP) (axin binding) repeat at codon 1580 (122) and three

20-amino acid repeats (20AARs) involved in β -catenin binding and degradation. The great majority of pathogenic APC mutations truncate the protein before the first SAMP repeat and leave a stable, truncated protein that encodes 0–3 20AARs.

It had been known for some years that somatic APC mutations tend to occur in a restricted region of the gene mutation cluster region (MCR) that lay approximately between codons 1250 and 1450 (123). In particular, it has been shown for both sporadic tumors and tumors in FAP patients that, when the first mutation is in the MCR, then the second event leads to loss of the normal function of the APC gene is due to loss of heterozygosity (LOH), mainly as a result of mitotic recombination. On the other hand, when the first mutation is not in the MCR, then the second event is a further mutation in the MCR. This implies that there is a selective advantage for mutations in the MCR (121).

The APC gene has 15 exons and encodes a large protein (310 kDa, 2843 amino acids) that possesses multiple functional domains that mediate binding to a variety of intracellular proteins including β -catenin, γ -catenin, glycogen synthase kinase (GSK)-3 β , axin, tubulin, and end binding protein (EB1) (124). The best-characterized function of APC is as a scaffolding protein in a multi-protein complex and its activity is modulated by Wnt signaling (125). This complex regulates the phosphorylation of β -catenin and thus controls the amount of β -catenin available for transcriptional activation via T cell factor and lymphoid enhancer factor (TCF/LEF) transcriptions factors. Other proteins that are part of this complex include GSK-3 β , β -catenin, axin and several kinases and phosphatases. A number of reviews have described the details of this pathway

(125-127). Briefly, in the absence of extracellular Wnt signals, GSK-3 β in the APC- β -catenin-axin complex is active, phosphorylating its serine and threonine residues and all three of these proteins and increases their interaction. In this way, phosphorylated β -catenin is for subsequent degradation by ubiquitin-mediated proteolysis. Downregulation of β -catenin is dependent on the presence of at least three of the seven 20AARs.

Truncated mutations of APC that lack all or most of the 20AARs are unable to bind β -catenin. Therefore, β -catenin cannot be phosphorylated and broken down. Accumulation of β -catenin within the cellular cytoplasm and nucleus associates with members of the TCF/LEF transcription factors and activates the expression of a variety of genes that can change the proliferation and differentiation state of cells, including those encoding cyclin D1, c-myc, and c-jun (128-130). However, there are additional pathways that contribute to the regulation of β -catenin and are independent of APC and other proteins involved in the Wnt pathway (131). These include the p53-inducible Siah-1 protein (132) and a retinoid X receptor (RXR) (133). Besides the Wnt pathway, other functions of APC are also involved in colorectal carcinogenesis.

APC mutation can affect actin cytoskeletal integrity, which is necessary to maintain the shape and adherence junctions of cells. The link of APC with actin is through β -catenin. β -catenin establishes this link by providing a bridge to α -catenin (134). The imbalances in actin cytoskeletal integrity can disturb cell-cell adhesion and cell migration, which play a role in the initiation of colon cancer.

Interaction of APC with β -catenin and adhesion complex has been implicated in cell-cell adhesion (135). The accumulation of β -catenin caused by truncated APC protein can bind to the TCF/LEF transcription factor, and down-regulate the transcription of E-cadherin gene expression. These complex interactions may finally result in the reduction in E-cadherin-mediated cell-cell adhesion and proliferation of cells (136, 137).

Another important role for APC is in cell migration. Colonic epithelial cells, derived from a stem cell, divide in the lower two-third of the crypts and migrate rapidly to the surface to form a single layer. The function of a wild-type APC is necessary in maintaining the direction of upward movement of these cells along the crypt-villus axis. Loss of wild-type APC functions affects cell migration. These cells, instead of migrating upwards towards the gut lumen, migrate aberrantly or less efficiently towards the crypt base where they accumulate and form polyps (138). The mechanisms by which APC might be involved in cell migration can be understood by its association with microtubules, EB1, IQ motif containing GTPase activating protein 1 (IQGAP1), APC-stimulated guanine nucleotide exchange factor (ASEF) and kinesin superfamily-associated protein 3 (KAP3) (139). For example, an interaction of APC has been shown with ASEF that may regulate the actin cytoskeletal network (140). Another example is APC binding with ASEF and controls its activity. ASEF is activated in colorectal cancer cells containing truncated APC. Active ASEF decreases E-cadherin-mediated cell-cell adhesion and promotes inappropriate cell migration.

APC is also a cell cycle regulator. Over-expression of APC is capable of

blocking cell cycle progression from G0/G1 to S phase (141). Matsumine's study (142) indicated that APC-DLG complex might participate in regulation of cell cycle progression, because the APC- β -catenin complex was shown to bind to the human homolog of the Drosophila discs large tumor suppressor (DLG) protein. This interaction required the carboxyl-terminal region of APC and the DLG homology repeat region of DLG. Ishidate stated that mutant APC lacking the carboxyl-terminal S/TXV motif for DLG binding exhibits weaker cell cycle blocking activity at G0/G1 phase than the intact APC (143).

APC may play a role in regulating apoptosis. Venesio et al. reported that a germline mutation of APC on codon 1383, in the 20AARs of the β -catenin binding domain, found in some FAP patients resulted in decreased apoptotic level. This decrease is independent of a somatic alteration on the wild type allele (144). These findings suggest that mutations in critical sites of the β -catenin binding domain of APC gene can alter the apoptotic surveillance rather than enhancing the β -catenin-TCF/LEF transcription of growth-promoting genes. The regulation of apoptosis by APC may be an important part of APC's tumor suppressor function. However, the mechanism responsible for the regulation of APC-induced apoptosis is not understood, but several recent evidences support the involvement of clusterin (145) and survivin (146). Wnt signaling pathway may also be involved in the increased apoptosis induced by APC protein. Survivin, which is downregulated in response to APC expression, is also downregulated by dominant negative TCF4 (146). This finding supports the idea that survivin is inappropriately activated by β -catenin/TCF/LEF transcription in

colon cancer.

β-catenin (CTNNB1)

β-catenin is a member of the APC/β-catenin/TCF/LEF pathway that plays an important role in the formation of colorectal cancer. Mutation of β-catenin often renders β-catenin insensitive to APC/β-catenin/GSK-3β mediated degradation. The majority of CTNNB1 mutations are missense mutations in the highly conserved exon 3, encoding for the GSK-3β phosphorylation region of β-catenin, result in the impairment of the ability of GSK-3β to phosphorylate β-catenin (147). Therefore, cancers with CTNNB1 mutations have increased β-catenin/TCF/LEF mediated transcription, which leads to the overexpression of genes such as c-myc and cyclin D1 (130, 148).

Accumulating findings support the role of β-catenin in colon carcinogenesis (149-153). Roh et al. (150) reported that antisense-mediated down-regulated β-catenin expression in APC-mutant human colon carcinoma cells inhibits the in vitro cell proliferation and cellular invasiveness. Wang's study (151) showed that in excessive cytosolic β-catenin, colonic cells become resistant to inflammatory factor nitric oxide-induced, p53-mediated apoptosis and survives with damaged DNA. This study suggested mutant β-catenin might contribute to human colonic carcinogenesis and tumor progression. In 1999, Harada et al. (149) constructed mutant mice that carried a dominant and stable β-catenin mutation, which was expressed in the intestinal and colonic epithelia. These mice developed numerous intestinal adenomatous polyps and some colonic microadenomas, which is similar

with a Min mouse phenotype. These results provide direct experimental evidence for the involvement of CTNNB1 mutations in Wnt signaling in colon tumor formation.

p53

Tumor suppressor genes are a class of genes with normal function that appear to be related to the control or suppression of cell proliferation. The inactivation of these genes caused by mutations resulted in the loss of normal cell growth control.

The tumor suppressor gene p53 is absent or mutated in 40%-50% sporadic colorectal cancers and 85% of all human colon cancers (154). This defect in p53 tends to be a later event in colorectal carcinogenesis. Fazil et al. stated that the role of p53 might be the induction of apoptosis in response to mutations of APC, ras and other genes. Therefore, p53 is inactivated at a late stage of tumor development (155). p53 gene is located at 17p13.1. Wild-type p53 is currently believed to be involved in maintaining genomic stability through the control of cell cycle progression and apoptosis in response to the genomic stress (156). Its expression level is very low in cells until it is activated by DNA damages such as ultraviolet irradiation or chemotherapeutic agents. Several studies suggested that wild-type p53 could induce apoptosis in colorectal cancer cells (157-160). Shaw's study (157) revealed that cells expressing wild-type p53 developed morphological features of apoptosis. DNA from both attached and detached human colon-derived cells was degraded into a ladder of fragments. Further molecular biological studies show that p53 levels are suppressed by Bcl-2 (159).

Additionally, a recent study suggests that p53 stimulates apoptosis by suppressing the level of survivin (160).

K-ras

K-ras is the best-studied and most common oncogene involved in colorectal carcinogenesis (161). K-ras mutations are detected in 58% of larger adenomas and 47-50% of colorectal cancers (162). The majority of K-ras point mutations occur at codons 12, 13, and 61 (163, 164). Mutated K-ras appears to be capable of stimulating Wnt signaling in colon cancer through suppression of GSK-3 β (165), as well as RAF/MAPK, JNK, and phosphotydylinositol 3-kinase (PI3-kinase), leading to constitutive growth promotion.

Another oncogene, cellular Src (c-Src) is a non-receptor protein tyrosine kinase whose activation is reported as an early event in the development of pre-neoplastic colonic adenomas and also detected in >70% of colon carcinomas (166). In metastasis of experimental colon epithelial cells, over-expression of c-src plays an important role by activating Akt/PKB-mediated survival pathways, which is leading to cell hyperproliferation (167).

3.2.2 Microsatellite instability (MSI) pathway

MSI is characterized by expansions or contractions in the number of repeats of simple DNA sequences (microsatellites). It has been identified in colorectal cancer associated with hereditary nonpolyposis colorectal cancer (HNPCC) syndrome (168) and DNA mismatch repair (MMR) enzymes, including hMSH2,

hMLH1, hPMS1, hPMS2, and hMSH6, have been shown to be responsible for MSI.

Mutation Mismatch Repair genes (MMR)

The DNA mismatch repair system (MMR system) consists of a complex of proteins that recognizes and repair base pair mismatches that occur during DNA replication. Inactivation of the MMR system due to germline gene defects accounts for the colon cancer family syndrome, HNPCC (169). HNPCC accounts for about 5%-7% of cases of colorectal carcinoma (170). The resulting colon cancer displays the phenotype of MSI, which is characterized by the size variation of microsatellites in tumor DNA as compared to matching normal DNA.

There are at least six different proteins involved in the MMR system have been identified in HNPCC. These proteins are hMSH2, hMSH3, hMSH6, hMLH1, hPMS1 and hPMS2. Recently, germline mutations in hMSH2 and hMLH1 genes have been suggested to cause early onset in colorectal cancer patients (171), and have been found in 45%-70% of HNPCC (172, 173). The germline mutations that occur in hMSH2 and hMLH1 are widely distributed throughout the gene. The mutations that occur in either gene tend to be point mutations that are single base-pair substitutions, deletions, or insertions (174). Study of the biochemistry of the MMR proteins has revealed that hMSH2 forms a heterodimer with either hMSH6 or hMSH3 and binds to the mismatch site. The complex of hMSH2 with hMSH6 is required for the correction of single-base mismatches. The complex of hMSH2 with hMSH3 is required for the correction of insertion-deletion loops (175).

Mutations in the hMLH1 and hPMS2 have been found in about 90% of HNPCC cases. Mutations in other MMR genes have been less frequent in HNPCC patients (169).

Mutation rate in cells with MMR deficiency are 100-1,000-fold greater than in normal cells, and the targeted genes particularly show instable microsatellite repeats in their coding regions (176). Indeed, many genes that possess microsatellite repeats are observed to be mutated in MSI colon cancers. Although MSI and the subsequent target gene mutations appear to occur throughout the adenoma-carcinoma progression, the precise stage of tumorigenesis in which mutation occurs is not clear. There are many targets of MMR gene inactivation. They include TGF- β receptor type 2 tumor suppressor gene (TGFB2), insulin like growth factor 2 receptor (IGFIR), Bcl-2-associated X protein (BAX) and caspase 5, the proteins that regulate apoptosis; E2F4 and TCF4, transcription factors; MSH3 and MSH6, DNA mismatch repair proteins; RIZ, the retinoblastoma protein-interacting zinc finger gene; and CDX2, an intestinal homeobox factor (177-181).

TGFB2

As much as 90% of the colorectal cancers with MSI have a TGFB2 gene mutation. One mutation found in the TGFB2 gene is that add or delete one or two adenine bases within or from 10 base-pair poly(A) repeat in the cysteine-rich coding region (codons 125–128). The other mutation in the TGFB2 gene is located at poly(GT)₃ with insertion of an extra GT in this region (177, 182).

These mutations produce truncated proteins that lack the cytoplasmic domain. In fact, alterations of TGFBR2 have been identified in up to 30% of colon cancers and are the most common mechanism identified to date for inactivating the TGF β signaling pathway (177). It is known that TGF β is a multifunctional cytokine that can induce growth inhibition, apoptosis, and differentiation in intestinal epithelial cells through regulating Type 1 plasminogen inhibitor (PAI-1) and cyclin dependent kinase inhibitor p15 genes expression. PAI-1 is the primary inhibitor of tissue type plasminogen activator (tPA) and urokinase-type 1 plasminogen activator (uPA), the products of which are implicated in the control of cell adhesion and invasion (183). Increased p15 expression, initiated by TGF β , can induce G1-phase cell cycle arrest (184). Thus, the loss of TGFBR2 can abolish TGF β -signaling and promote cell proliferation and development of colorectal cancer. In a study of colon cancer cell line HCT116 with a mutant TGFBR2 gene, investigators found that reconstitution of wild-type TGFBR2 suppressed the tumor phenotype of the cell line (185).

IGFIIR

One of the microsatellites in the coding region of IGFIIR gene is poly(G) repeat which is often mutated with 1- or 2-base pair deletions or insertions. These mutations produce frameshifts and premature stop codons (186). IGFIIR plays a critical role in cell growth, survival and differentiation as a tumor suppressor; however, once mutated such as the loss of IGFIIR function due to mutations in MSI cells may enhance its proliferation due to accumulation and activation of

IGFII and down-regulation of TGF β 1, which are cognate ligands of IGFIIR, and thus play a major role in tumorigenesis (187). Souza et al. (188) transfected a zinc-inducible construct containing the wild-type IGFIIR cDNA into colon cancer cell line SW48, which has mono-allelic mutation in IGFIIR, and found that zinc induction of exogenous wild-type IGFIIR expression reproducibly decreased growth rate and increased apoptosis. These data prove that wild-type IGFIIR functions as a growth suppressor gene in colorectal cancer cells. A very recent study in APC (Min/+) mice (189) provides genetic evidence that the consequences of excess supply of IGF-II ligand can be rescued by soluble IGFIIR, and this supports the rationale application of this novel IGFII-targeted therapy for colon cancer.

SMAD4 (MAD homologue 4 (Drosophila))

Tumor suppressor gene SMAD4, also called DPC4 (deleted in pancreatic carcinoma, locus 4), is located at chromosome 18q where LOH occurs in approximately 70% of colon adenocarcinoma (190).

The SMAD proteins (R-SMADs) serve as intracellular mediators to regulate TGF β signaling. In brief, TGF β ligand binding with TGFBR2 recruits TGFBR1 into a tetrameric receptor complex which results in transphosphorylation and activation of TGFBR1. After phosphorylation, the TGFBR1 becomes an active kinase to phosphorylate SMAD 2 and SMAD3. Phosphorylated SMAD2 and SMAD3 allow the formation of homo- and heterodimerization complex, including SMAD4. This complex then translocates into nucleus, binds with DNA, and

stimulates the expression of target genes (191, 192). As SMAD4 is a key mediator of TGF β responses and can interact with all the R-SMADs, it is not surprising that SMAD4 alterations have been found in up to 16% of colon cancer (193). Pholnukulkit and coworkers (194) investigated the importance of SMAD4 in TGF β signaling pathway. Their study suggested that deletion of SMAD4 leads to a defect of formation of SMAD2/SMAD4 complex upon activin A (TGF β family member) stimulation in HT-29 colon cancer cell line. It was also shown by Schwarte-Waldhoff et al. that re-expression of SMAD4 in SW480 colon carcinoma cells could suppress the tumor formation in nude mice, which provides definite proof for its tumor suppressor (195). However, they further reported that expression of SMAD4 did not affect growth rates of SW480 cells *in vitro*. Since SW480 cells endogenously express high levels of TGF β (196), their findings indicated that SMAD4 reconstitution initiated other pathways to impede tumorigenesis rather than restored a TGF β induced growth inhibitory effect. TGF β independent way of SMAD4 mediated tumor suppression may be involved in transcriptional regulation of E-cadherin (197), uPA and PAI-1 genes (85). These findings suggested a potential role for SMAD4 as a modulator of cell adhesion and invasion.

3.3 Epigenetic alterations in colorectal cancer

Epigenetic changes are modifications of the genome heritable during cell division that do not involve a change in DNA sequence (198). Epigenetic dysregulation has been increasingly recognized as an important mechanism in

tumorigenesis since its discovery in human tumors in 1983 (199, 200). This dysregulation includes hypomethylation, which leads to oncogene activation and chromosomal instability, hypermethylation and tumor suppressor gene silencing, and chromatin modification acting directly and cooperatively with methylation to modify gene expression. In addition, disrupted genomic imprinting appears to contribute to colorectal cancer risk.

3.3.1 DNA methylation

DNA methylation is present throughout the majority of the genome and is maintained in relatively stable patterns that are established during development (201). In humans, approximately 70% of CpG dinucleotides are methylated. However, there are regions that contain higher proportions of CpG dinucleotides called CGIs, which are 0.2-3 kb long sequences and are composed of greater than 50% cytosines/guanines. They are present in the 5' region of approximately 50%-60% of genes and are normally maintained in an un-methylated state (202). In cancers, many of these CGIs become aberrantly methylated, and this aberrant methylation can lead to transcriptional repression.

Colon tumors exhibit hypermethylation of tumor suppressor genes (203). Previous studies indicate that DNA hypermethylation may occur not only secondary to a genetic predisposition in the colorectal carcinogenesis, but also are key pathogenic events in cancer formation that drive the initiation and progression of the ACF-adenoma-carcinoma sequence (204).

DNA hypomethylation is also found in both human and rat colon tumors. DNA

hypomethylation has been shown to increase chromosomal instability, to increase histone acetylation and to be associated with increased expression of growth-related genes in carcinogenesis (205, 206).

3.3.2 Epigenetic changes in Wnt pathway components

Transcriptional silencing of APC by promoter methylation has been suggested as an alternative to somatic mutation in colorectal tumorigenesis. Recently, the methylation of CpG sites in the promoter region of APC gene has been reported in different types of cancers, including colorectal cancer (207-209). Esteller et al. (208) analyzed the APC promoter methylation in human colorectal cancer. They reported that the APC promoter is hypermethylated in 18% of primary sporadic colorectal carcinomas (n=108) and adenoma (n=48), and neoplasia with APC methylation fails to express the APC transcript, suggesting that it is an early event in colorectal carcinogenesis. Deng et al. (210) has shown convincingly that methylation leads to reduction or loss of APC expression. In this study, they observed the methylation status of APC promoter in 22 colorectal cancer cell lines with different APC expression levels. They found that the methylation of CpG sites was invariably correlated with the loss of gene expression. By nuclease accessibility assay, they also observed a correlation between the closed chromatin conformation in APC promoter and loss of gene expression. When the low-expressing or non-expressing cell lines were treated with a DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-aza-dC), the APC expression in these cells was induced, CpG sites were demethylated, and closed

chromatin conformation was opened.

Several other studies have reported methylation of the promoters of other Wnt pathway members, including Dickkopf-1 (DKK1) (211), Wnt inhibitor factor-1 (WIF1) (212) and secreted frizzled-related proteins (SFRPs) (213). These three family members are distinct classes of extracellular Wnt antagonists and may be expected to counter persistent tumorigenesis stimulated by Wnts. DKK-1 is a candidate gene for hypermethylation-associated transcriptional inactivation in human cancer since a 5'-CpG island is located around its transcription start site and it has been previously reported to be downregulated in human colon cancer (214). DKK-1 gene is transcriptionally silenced by CpG island promoter hypermethylation in colon cancer cell lines, whereas treatment with the DNA-demethylating agent 5-aza-dC restored DKK-1 expression (211). Similar experiments have been performed in analyzing the association of methylation level and WIF-1 mRNA expression (213). An increased methylation of the promoter region was demonstrated in a cohort of 51 advanced colorectal cancers. Caldwell et al. (215) reported that hypermethylation was identified in 82% cancers and in only 30% matched normal mucosal samples ($P < 0.001$), comparable with the 95% found by Suzuki et al. Reduced transcript levels were also seen in >75% of these cases. The best evidence that methylation changes have functional consequences comes from study of the WIF-1 (216). Downregulation of WIF-1 expression was observed in 72.7% of 44 colorectal adenoma tissues and 78.2% of 23 early mucosal or submucosal colorectal carcinoma tissues. Furthermore, no significant association between WIF-1

downregulation and clinicopathological characteristics was found, suggesting that downregulation of WIF-1 expression is an early event in carcinogenesis of these cancers (216). Treatment with demethylating agent, 5-aza-dC, restored WIF-1 expression in colon cancer cell lines. Transfection of the WIF-1 gene construct into SW48 colon cancer cell line lacking WIF-1 expression resulted in a significant inhibition on colony formation and cell proliferation.

3.3.3 Epigenetic changes in other tumor suppressor genes

Inactivation of hMLH1 presumptively plays an initiating role in the pathogenesis of MSI colon cancers. Accumulating evidence suggest that aberrant methylation exists in the promoter of hMLH1 in sporadic MSI colon cancers, and the hMLH1 expression can be restored by demethylating the hMLH1 promoter in cell lines (217, 218). Structure analysis of the methylation status of specific CpGs in the hMLH1 promoter has shown that the methylation status of small clusters of CpGs in the 5' region of the hMLH1 promoter appears to dictate the transcriptional status of the gene through interfering with CBF transcription factor binding to a CCAAT box (219), resulting in decreasing downstream genes expression. Moreover, the aberrant hypermethylation of 5' CpG dinucleotides could silence a variety of tumor suppressor genes including CDH1 (E-cadherin), p16, etc. Grady et al. (220) demonstrated that loss of expression of CDH1 concurrent with CpG methylation of the wild type CDH1 allele in tumors that occur in the setting of hereditary diffuse gastric cancer. However, more recent study questioned the role of CDH1 hypermethylation in colon cancer initiation.

Kim et al. (221) measured the methylation status of eight genes from adenomas to adenomacarcinomas, including CDH1. Unexpectedly, CDH1 methylation decreased with tumor progression (chi-square test for trend, $p=0.023$). Another tumor suppressor gene that has been studied is p16^{INK4a}. Although mutation of p16^{INK4a} has not been described in colon cancer, methylation of p16^{INK4a} is detected in 18% (222) to 50 % (223) of colon cancer and 75% colon cancer cell lines (224), and has been found not only in colon cancer, but also in adenomas (225). This observation demonstrates that aberrant promoter methylation is occurring early in the adenoma sequence.

3.3.4 Folate and colorectal carcinogenesis

Folate and one-carbon metabolism

One-carbon metabolism is a network of interrelated biochemical reactions in which a one-carbon unit is transferred to tetrahydrofolate for subsequent reduction or oxidation (226). The transfer of this one-carbon moiety into biochemical pathways is essential for DNA synthesis and DNA methylation (Fig. 2). 5, 10-methylenetetrahydrofolate, an intracellular form of folate, is required for conversion of deoxyuridylate to thymidylate and can be oxidized to 10-formyltetrahydrofolate for purine synthesis. Thus, folate is an important cofactor for DNA synthesis. Folate also plays an essential role in one-carbon transfer involving remethylation of homocysteine to methionine, thereby ensuring the provision of S-adenosylmethionine (SAM), the primary methyl group donor for most biological methylation reactions (227). After transfer of the methyl group,

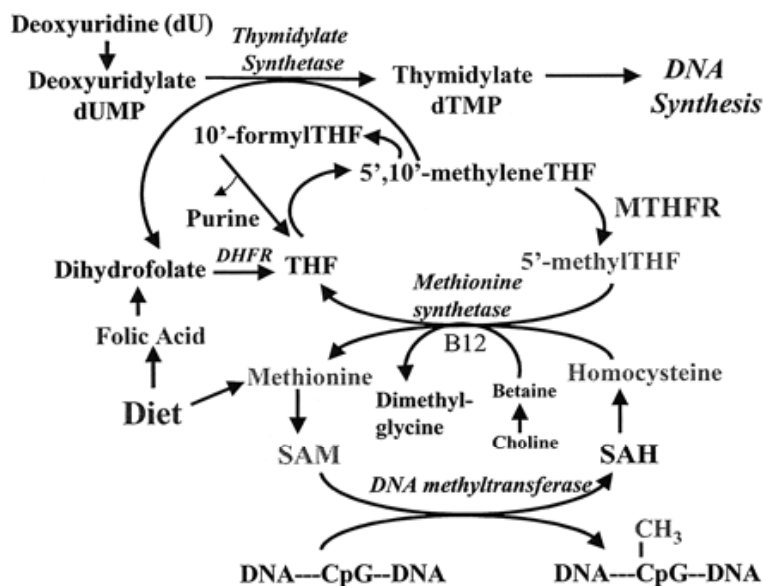


Fig. 2. Simplified scheme of folate metabolism involving DNA synthesis and methylation (227)

SAM is converted to S-adenosylhomocysteine (SAH) by DNA methyltransferases. Methyl group is then transferred to DNA sequence.

Folate deficiency and colorectal carcinogenesis

Folate deficiency has been associated with colorectal cancer risk based on the biochemical function of folate in mediating one-carbon metabolism. In 1992, Cravo (228) first proposed that folate deficiency enhances colorectal carcinogenesis because of evidence from animal experiments that demonstrated altered folate status could affect genomic DNA methylation. However, the effect of isolated folate deficiency on DNA methylation in the colorectum has not yet been clearly elucidated. In rodent studies, folate deficiency in conjunction with a colorectal carcinogen (dimethylhydrazine (DMH) or azoxymethane (AOM)) failed to induce significant genomic DNA hypomethylation in rat colon (229-231). On the other hand, several studies reported that folate deficiency could

diminish the occurrence of colorectal cancer, which may be attributed to the known role of folate in cell multiplication (231, 232).

Accumulating evidence over the past decade suggests that folate status (assessed by dietary folate intake or by the measurement of blood folate levels) is inversely related to the risk of sporadic and ulcerative colitis-associated colorectal cancer (CRC) or its precursor, adenoma (233-235). In some human intervention studies, folate supplementation increased the extent of colonic genomic DNA methylation in subjects with colorectal adenoma or cancer (236, 237).

In conclusion, the effect of folate deficiency on DNA methylation in the colon in animal studies are inconsistent and do not support the hypothesis that folate deficiency induces DNA hypomethylation in the colon, whereas folate supplementation appears to be capable of increasing the degree of DNA methylation in the colorectum in some human studies. Therefore, the potential for folate to modulate DNA methylation and thus modify colorectal cancer risk still remains reasonable in biological level and is worthy of future studies.

4. Models of colorectal cancer

Animal models have contributed significantly to understanding of the carcinogenesis and the environmental factors involved in the pathogenesis of colon cancer (238). The ideal animal model should mimic the human disease in terms of morphology, biochemical alterations, and biological behavior (239). Unfortunately, although each animal model can approximate part of the characteristics of human CRCs, they all have limitations. A meta-analysis showed that the effects of aspirin, β -carotene, calcium, and wheat bran were not strikingly different in humans and animal models. However, some chemopreventive agents cannot produce the same effect in animals and in humans (240). The advantages and limitations of these animal models are addressed below.

4.1 Models of chemically induced colorectal cancer

Carcinogens can induce colorectal cancer in rats and mice. Frequently used carcinogens are dimethylhydrazine (DMH) and its metabolite azoxymethane (AOM), N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and N-methyl-N-nitroso-urea (MNU) (241). Carcinogens can be administered via four routes: oral, subcutaneous, intrarectal and intramuscular. The incidence of CRC development depends on the carcinogen used, the dosage, the duration and frequency of administration, and the routing and timing of administration. Kobaek-Larsen et al. (242) states that intrarectal administration of DMH or AOM or MUN in rats is much more effective than oral dosing in the development of colorectal cancer. Additionally, the sex, age and genetic background of the mice can affect the

incidence of CRC development. Moreover, the effective concentration of these carcinogens can be influenced by the intestinal flora, the diet and the immunological status of the animals (241).

Many investigators use AOM to induce colon tumors and to study the effects of nutritional factors and chemopreventive agents. AOM has been demonstrated to be a potent inducer of carcinomas of the large intestine in various strains of male and female rats. Endoscopic examination of animals treated with AOM revealed that the first visible colon tumors can be detected 15 weeks after treatment, and that the mean latency period of such tumors is about 20 weeks (242). AOM-induced tumors share many histopathologic characteristics with human tumors, and follow the ACF-adenoma-carcinoma sequence. One of the characteristics of the human disease process reflected in the AOM-rat model is the occurrence of both adenomas and adenocarcinomas. Reddy's study (238) indicated that in AOM-rat model, about 70% of colon tumors are adenocarcinomas, and the rest are adenomas. Like human colon tumors, enhanced cyclooxygenase-2 expression was observed in AOM-induced colon tumors (243). AOM treatment also induces K-ras mutation (30–60%) and β -catenin mutation (244-246), but, unlike human tumors, they seldom have Apc mutations (<15%) (240, 245), and never exhibit p53 mutations.

4.2 Models of genetically mutant induced colorectal cancer

Mice homozygously deleted for Mlh-1 or Msh-2 develop lymphomas but are also prone to intestinal neoplasia and therefore represent a good model for

studying HNPCC development. $Mlh1^{-/-}$ mice 6 months or older develop gastrointestinal tumors in 33% of cases. Moreover, addition of an *Apc* gene mutation into the $Mlh1^{-/-}$ mice resulted in 100% GI tumor formation (247). These model reveal that exposure of MMR-deficient cells to endogenous mutagens may develop HNPCC.

Mice exhibiting *Apc* gene mutations have been used to study the general principles of carcinogenesis. The multiple intestinal neoplasia (Min) mouse, which carries a fully dominant mutation converting codon 850 of the murine *Apc* gene from a leucine to a stop codon, resulting in premature truncation of the *Apc* protein, was first reported by Moser et al. (248). This is similar to germline mutations in the APC gene in humans with FAP and sporadic cancers. On the C57BL/6J (B6) background, Min mice develop more than 50 adenomas throughout the intestinal tract and rarely survive beyond 120 days (248). Min mice also develop a variety of lesions in other tissues including desmoid tumors, epidermoid cysts and mammary tumors. This reflects part of the spectrum of lesions seen in humans with mutations in the APC gene (249). However, ACF and adenocarcinomas are not or seldom observed in this model. Consequently, the ACF to carcinoma progression is not established in this model (240, 245). Moreover, K-ras and p53 mutations are not detected in Min mice polyps, in contrast with human tumors (250), and p53 inactivation does not increase tumor number in Min mice (251). In addition, similar to humans, methylation plays a role in Min mice carcinogenesis, since a reduction in DNA methyltransferase activity suppresses polyp formation (15). After the Min mice discovery, other

mice have also been genetically modified on Apc with truncations in positions 580, 716, 1309, or 1638 (245). Different mutations lead to different phenotypes, similar to the human condition. The average polyp number in Min mice is 40 ± 20 , which is six times fewer than Apc716 mutant mice. Apc 1309 and 1638 mice have 30 and 3 polyps respectively (245).

Although Apc mutant mice are promising models for human CRC, the major drawback is that tumors occur predominantly in the small intestine and a few in the colon. In contrast, human tumors frequently exist in the colon. This discrepancy may be explained as a result of differences in key enzymes between the small and large bowel of Min mice. For instance, phospholipase A₂ and cyclooxygenase-2 (COX-2) are upregulated in the colonic tumors of human and rat (253), but also in small intestinal tumors of Min mice (254, 255). The resulting increase in prostaglandin E₂ (PGE₂) level would promote cancer growth. Therefore, Min mice have more polyps in the small intestine. Another explanation is polyamine levels are lower in the colon than in the small intestine of Min mice (256, 257). The colorectal mucosa of individuals with FAP contains elevated levels of the proliferation-associated polyamines. In Min mice, mRNA content of ornithine decarboxylase (ODC), the first enzyme in polyamine synthesis, is increased in both the small intestine and colon; while mRNA content of antizyme (AZ), a protein which negatively regulates ODC, is decreased significantly in the small intestine but not the colon (257). Consistent with the changes in ODC and AZ gene expression, small intestinal, but not colonic, polyamine content was elevated in Min mice compared with normal littermates. Treatment of the Min

mice with ODC inhibitor, difluoromethylornithine (DFMO), decreased small intestinal, but not colonic tumor number. Therefore, this low level of polyamines in the colon may explain why Min mice have few polyps in the colon.

5. Obesity and colorectal cancer

5.1 Yellow obese mouse model

The *agouti* gene has been mapped and identified in a number of species (258, 259). Cloning and sequence analysis revealed that the mouse *agouti* gene is located in the distal region of chromosome 2 and encodes for a small-secreted protein. In wild-type mice, *agouti* is only expressed in the skin during the hair growth cycle (260). The human homologue of *agouti* is located on chromosome 20 and encodes a 132 amino acid protein, agouti signal protein (ASIP) (261). Despite the high degree of protein homology (85% of amino acids identical to mouse) the expression pattern is different. Whereas mouse *agouti* is only transiently expressed in the hair follicles, human *agouti* is expressed in diverse tissues; primarily adipose tissue followed by testis, heart, ovary and at low levels in liver, kidney and foreskin (261, 262).

The mechanism of agouti action on coat color is based upon antagonism of melanocortin receptors, resulting in a shift from eumelanin to pheomelanin synthesis within the hair follicle. α -melanocyte stimulating hormone (α -MSH) binds to its receptor, melanocortin 1 receptor (MC1-R), which is coupled to the heterotrimeric guanine nucleotide binding proteins that activate adenylate cyclase. The resulting increase in intracellular cAMP levels leads to the activation of the rate limiting enzyme in melanogenesis, tyrosinase. Agouti antagonizes the binding of α -MSH to MC1-R, thereby decreasing the overall rate of melanogenesis and increases the incorporation of sulfhydryl compounds into dopaquinone to produce yellow pigment (263-266).

Two of the dominant agouti mutations have been studied extensively, namely, “lethal yellow” (A^y), first described by Cuenot (260), and “viable yellow” (A^{vy}), first described by Dickie (266). The lethal yellow mutation is characterized by prenatal lethality of the homozygous A^y/A^y genotype. The viable yellow $A^{vy}/-$ phenotype differs from that of the clear yellow $A^{vy}/-$ phenotype by exhibiting eumelanic mottling, i.e., irregular areas or small spots of agouti/black hair on a yellow background (267). Molecular definition of these mutations was first identified at the Oak Ridge National Laboratories (260). Briefly, A^y is characterized by a 120-170kb deletion that includes the entire coding regions of the *Raly* (heterogeneous nuclear ribonucleo-protein associated with lethal yellow) gene as well as the non-coding exon of agouti. As a result, the A^y allele is under the control of the *Raly* promoter, which is normally constitutively expressed in all somatic cells (268). Therefore, the *Raly* promoter overrides the agouti gene’s promoter in regulating its transcription and thus induces formation of yellow pigment by the hair follicle melanocytes. The embryonic lethality, which is associated with the A^y/A^y genotype, may actually result from the absence of *Raly* expression in the developing embryo (267). The ubiquitous over-expression of the *agouti* gene is also the basis to the syndrome expressed by $A^{vy}/-$ mice. It is due to insertion of an intracisternal A particle (IAP) into noncoding exon 1A (269).

Transgenic mice ubiquitously over-expressing agouti become obese, develop diabetes and tumors (270). *Agouti* expression in skin has little effect on the obesity phenotype. It is likely that Mc4r and Mc3r are responding to ectopic agouti protein expression, resulting in weight gain. Mc4r is expressed in the

hypothalamus and plays a role in the regulation of feeding and metabolism (271). Administration of a Mc4r agonist inhibits feeding and administration of a Mc4r antagonist increases feeding suggesting that agouti protein, an antagonist of Mc4r may play a role in *agouti*-induced obesity (272). Mouse agouti protein also antagonizes Mc3r. *Mc3r* knock-out mice have increased fat mass, reduced lean mass and a greater feeding efficiency compared with wild-type mice (273). Ectopic expression of *agouti* protein in adipocytes may lead to an inhibition of lipolysis and stimulation of lipogenesis. Data from Moustaid and colleagues (274) suggest that lethal yellow mice have increased levels of fatty acid synthetase (FAS) and stearoyl-CoA desaturase (SCD), which are involved in fatty acid synthesis and desaturation of saturated fatty acids, relative to lean controls. Treatment of 3T3-L1 adipocytes with recombinant agouti protein increased FAS and SCD mRNA levels 1.5 and 4 fold, respectively (274). Adrenocorticotrophic hormone (ACTH) is a potent lipolytic hormone. In mouse adipocytes (275), ACTH can bind to Mc2r and elevate cAMP, resulting in stimulating lipolysis (275). Therefore, agouti protein antagonism of ACTH to adipocytes may lead to an inhibition of lipolysis. More recently, agouti has been shown to up-regulate adipocyte transcription factors, signal transducers and activators of transcription (STAT1 and STAT3) and peroxisome proliferators activated receptor (PPAR)- γ protein in 3T3-L1 adipocytes and in transgenic mice expressing *agouti* in adipose tissue (272, 276).

The divergence of in expression patterns between mice and humans suggests that agouti may have functions other than pigmentation in humans. The link

between *agouti* expression in human adipose tissue and obesity/type2 diabetes was investigated. Voisey's study (277) indicated that *agouti* mRNA was negatively correlated with BMI in men but not in women. Smith and coworkers (278) did experiments with cultured human pre-adipocytes and cells obtained from transgenic mice. They found that over-expressing *agouti* could regulate both pre-adipocyte proliferation and differentiation through melanocortin receptor (MCR) signaling in adipose tissue, which demonstrated that *agouti*/melanocortin system is a potent regulator of adipogenesis in humans.

5.2 Summary of epidemiologic evidence of obesity and CRC

The relationship between excess body weight and the incidence of colon cancer has been well established in epidemiological studies (279-283). In 1992, Lee and Paffenbarger (282) reported that obesity in adolescence was found to be strongly related to colonic cancer. Similar relationships are also seen for colon adenoma, with stronger associations for larger adenomas (283). Four years ago, the International Agency for Research on Cancer (IARC) published a comprehensive evaluation of the available literature on weight and cancer. Their report concluded that obesity had been consistently associated with higher risk of colorectal cancer in men (relative risks of approximately 1.5-2.0) and women (relative risks of proximately 1.2-1.5) in both case-control and cohort studies (284). However, there is a different association of body weight with colon cancer risk between men and women. The BMI has been found to be positively related to the risk of colon cancer in men (284), whereas this association is less consistent and weaker for

women (285-287). One reason for the discrepancy is that men and women have the different body compositions. Support for this, two recent cohort studies found that waist circumference and waist-to-hip ratio (WHR), indicators of abdominal obesity, were strongly associated with colon cancer risk in both men and women (288, 289), which indicate that abdominal obesity is a risk factor for colon cancer in both sexes. Another explanation for sex difference may be related to the differences and changes in estrogen levels in women. The modifying effect of estrogen suggests that estrogen may enhance the association between BMI and colorectal cancer (290). Hormone Replacement Therapy (HRT) use has been associated with reduced risk of colon cancer (291, 292). A meta-analysis of 18 observational studies of colorectal cancer and HRT also indicated a 20% reduction in colon cancer among ever users compared with never users (RR=0.80) and a 34% reduction among current users (RR=0.66) (293).

5.3 Current hypothesis for correlation between obesity and CRC

Adipose tissue is an active endocrine and metabolic organ that can have effects on the energy balance and lipid metabolism of other tissues. It also regulates their physiology through the release of hormones such as leptin, adiponectin, resistin and tumor necrosis factor- α (TNF- α).

5.3.1 Chronic hyperinsulinaemia

Recently, Giovannucci (294) outlined a mechanism by which elevated BMI may influence colon cancer risk and suggested that altered glucose-insulin

dynamics may be involved. Excess weight results in increased release of free fatty acids, resistin and TNF- α and reduced release of adiponectin by adipose tissue, which develops insulin resistance, a metabolic state characterized by reduced insulin sensitivity in liver, skeleton muscle and other tissues. Chronically increased insulin may exert a tumorigenic effect which is mediated by binding to insulin receptors in the neoplastic cell surface or by the changes from hyperinsulinaemia. Secondary to hyperinsulinaemia, insulin promotes the synthesis and biological activity of insulin-like growth factor 1 (IGF-1) that regulates cellular proliferation. In addition, insulin has effects on the synthesis and biological activity of sex hormones (estrogen, androgens and progesterone) that also play a role in the association between obesity and CRC, as discussed in a subsequent section (5.3.2 IGF-1 and IGFBP).

In vitro studies demonstrated that insulin stimulates growth of normal colonic and carcinoma cells (295-297). Similarly, cancer-enhancing effect of insulin has been demonstrated in animal models in which either animals are treated with insulin injections (298) or fed a high energy, high fat diet that lead to insulin resistance (299).

Epidemiological evidence that insulin is associated with colorectal cancer (CRC) risk is showing a relationship between type 2 diabetes and especially visceral obesity and CRC. The major environmental determinants of type2 diabetes include high body mass index (BMI), increased central obesity, physical inactivity, and excessive intake of energy and dietary patterns that stimulate secretion of insulin. These factors are remarkably similar to the constellation of

risk factors emerging for colon cancer. In one large prospective study of women (300), diabetic women had a 43% increased risk of colorectal cancer and a 49% increase in colon cancer compared with non-diabetic women. Another recent study found that women with a type 2 diabetes history were at increased risk of colorectal cancer (RR=1.55, 95%CI=1.04-2.31) (301).

5.3.2 IGF-1 and IGFBP

The principle stimulus for the synthesis of IGF-1 is in the liver, which is the source of over 80% of circulating IGF-1. Because more than 90% of IGF-1 is bound to IGFBP-3, little IGF-1 is circulating free. The synthesis and biological activity of IGF-1 is regulated by insulin. High levels of insulin in type2 diabetics cause an increase in hepatic growth hormone (GH)-receptor level, resulting in increases GH activity and increased synthesis and blood levels of IGF-1 (302). In addition, insulin reduces hepatic secretion of IGFBPs and inhibits IGF-1 action *in vitro* (303).

Epidemiological studies suggest that increased serum levels of IGF-1 and decreased levels of IGFBP-3 may be independently related to risk of colorectal cancer (304-306), suggesting that it is the ratio of IGF-1/IGFBP-3 that is relevant in relation to colorectal cancer risk. For example, in the Nurses' Health Study, three stages of colorectal carcinogenesis were examined in relation to base-line plasma IGF-1 and IGFBP-3 (305). Controlling for IGFBP-3 level relative to women in the low tertile of IGF-1, those in the high tertile were at elevated risk of colorectal cancer (RR=2.18; 95% CI, 0.94-5.08) and high-risk adenoma

(RR=2.78; 95% CI, 0.76-9.76). Controlling for IGF-1 level, women in the high tertile of IGFBP-3 were at lower risk of colorectal cancer (RR=0.28; 95% CI, 0.10-0.83) and high-risk adenoma (RR 0.28; 95% CI, 0.09-0.85). Neither IGF-1 nor IGFBP-3 had any appreciable relation to small, tubular adenomas. A very recent study concerning IGF-1, IGFBP-3 and adenoma risk indicated that ratio of IGF-1/IGFBP-3 and levels of IGF-1 are associated with adenomatous polyps in comparison with non-adenoma controls (307). However, a meta-analysis of IGF-1 and IGFBP-3 and colorectal cancer showed only a modest association with IGF-1 and no protection with IGFBP-3, and regression models could not confirm a dose-response association of IGF-1 and colorectal cancer (308). In a cohort of 14,275 women in New York, baseline IGF-1 and IGFBP were assayed from the serum of 102 women who subsequently developed colorectal cancer and 200 matched control subjects (309). Colorectal cancer showed a modest but positively increased risk with higher levels of IGF-1 but also an increased risk with higher levels of IGFBP-3. Thus, the association of IGF-1 with CRC has not been consistently observed.

The biologic basis for the role of IGF-1 in CRC pathogenesis is based upon IGF-1 regulation of cell proliferation, differentiation, metastasis and apoptosis (310, 311). The action of IGF-1 is initiated by binding to IGF-1 receptors. Both colorectal epithelia normal and cancer cells express IGF-1 receptors *in vitro*; when activated by IGF-1, the receptor-ligand complex inhibits apoptosis and allows progression through the cell cycle (312). Support for this is provided by Lahm's study (313), which indicated that stimulation by IGF-1 cause's

proliferation *in vitro* while blocking IGF-1 receptor inhibits growth of colorectal cancer cells. Further support for a link between the IGF-1 and cancer is that overexpression of IGF-1 receptors is critical for the survival of transformed cells (314). In addition, IGFBP-3, which can oppose those of IGF-1 in part by binding IGF-1 and thus reducing free IGF-1 levels, may act as an independent apoptotic agent (315, 316) and inhibit target cells. In tissues, IGFBP proteases can enhance IGF-1 availability by cleaving IGFBP, thereby increasing free IGF-1 concentration. Therefore, the biological activity of IGF is determined by the integrated actions of circulating IGF-1 and IGFBP and by local production of IGF, IGFBP and IGFBP proteases (316).

5.3.3 Estrogen

Clinical studies also indicate that the incidence of colon cancer is lower in women than in men (317), suggesting a role for estrogen in colon tumorigenesis. Estrogen acts by binding to the estrogen receptor (ER), a ligand-activated transcription factor that regulates transcription of target genes in the nucleus by binding to estrogen response element (ERE) regulatory sequences in target genes and recruiting co-regulatory proteins (CoRegs) such as coactivators (318). ER- β is the predominant ER form in both normal and malignant human colon tissue, as well as human colon cancer derived cell lines (319, 320). ER- β protein levels are reportedly lower in colon tumors compared with normal colon tissue, and loss of ER- β is associated with advanced stages of colon cancer and tumor cell dedifferentiation, suggesting a protective role for ER- β in colon tumorigenesis

(320, 321).

Adiposity influences the synthesis and bioavailability of endogenous estrogens (322). Adipose tissue expresses various sex-steroid-metabolizing enzymes that promote the formation of estrogens from androgenic precursors. In men and postmenopausal women, adipose tissue is the main site of estrogen synthesis, and BMI is directly related to circulating levels of estrone and estradiol (323).

5.3.4 Adipokines and CRC

Epidemiological studies have identified obesity as one of the major risk factors for cancer. A recent prospective study over a period of 16 years on 900,000 adults in the United States by Calle et al. (324) showed an association between increased BMI and risk of death from all cancers. The cross talk between cancer cells and adipose tissue has been increasingly accepted (325). The contributions may be due to the adipocyte-derived factors.

Leptin and CRC

Leptin is a highly conserved 16-kDa hormone and it is predominately expressed in adipose tissue. Leptin appears to play a role in regulating energy homeostasis, neuroendocrine and immune function (325, 326). A relationship between leptin signals and the pathogenesis of colon cancer was suggested in both mice and human studies. db/db mice have obese and diabetic phenotypes due to disruption of the leptin receptor. Recent studies using this genetic mouse model treated with azoxymethane, a powerful carcinogen that causes chemically induced

colon cancer, found that increased azoxymethane-induced pre-malignant colon lesions were detected in homozygous db/db mice compared to heterozygous or wild strains (327). The case-control studies showed that leptin is elevated in obese individuals (328, 329). In Statin's study (329), serum leptin levels were measured from men diagnosed with cancer of the colon or rectum after blood collection, and the controls matched with age and date of blood collection. The data showed an approximately 3-fold increase in colon cancer risk with increasing concentrations of leptin up to an odds ratio (OR) of 2.72 for top vs. bottom quartile. Moreover, energy restriction, a well-established protective factor against cancer, decreases leptin levels (330). However, one animal study did not support the role for leptin in carcinogenesis promotion (331); leptin administration to azoxymethane-induced Fischer 344 rats significantly inhibited ACF formation in the middle and distal colon compared with controls ($p=0.006$). That study provides the first evidence that leptin reduces the development of chemically induced precancerous lesions in colon. *In vitro*, leptin stimulated proliferation and invasiveness of human colon cancer cells (332-334). Leptin has also been demonstrated to stimulate angiogenesis, which is essential for tumor growth, invasion and metastasis (335).

Leptin acts by binding to its receptor. The presence of leptin receptor (Ob-R) has been demonstrated in normal, precancerous and malignant human colonic epithelium as well as in colon cancer cell lines. Activation of the leptin receptor has been reported to activate multiple signaling pathways in different system, but the mechanisms involved in the regulation of colonic epithelial function remain to

be determined. One study (336) indicated that the proliferative and anti-apoptotic actions of leptin in human colon cancer cells involved activation of JNK mitogen activated protein kinase (MAPK), JAK2 and PI3 kinase/Akt. Fenton (334) reported that leptin induced elevated IL-6 production in preneoplastic Apc^{Min/+} IMCE cells, and it was associated with STAT3, ERK, p38, MEK and JAK2 activation and associated STAT3 nuclear activation and translocation.

Adiponectin and CRC

Adiponectin is also called 30-kDa adipocyte complement-related protein, Acrp30, adipoQ, APM-1 and gelatin-binding-protein-29 and is the most abundant cytokine produced by adipocytes (337), with circulating levels approximately two to three times higher in females than in males (338, 339). Human adiponectin is a 274-amino acid and 30-kDa protein hormone and encoded by the gene APM1 located on chromosome 3q27 (340). Adiponectin consists of three domains including an N-terminal signal peptide, a collagen-like motif and a C-terminal C1q-like globular domain. At least two forms, that is low molecular weight (LMW) oligomers that is hexamers (two trimers) and high molecular weight (HMW) oligomers consisting of four-six trimers, exist in serum (341, 342). It has been suggested that the HMW adiponectin complex is the major source of the active form of this protein (342). Adiponectin is abundant in human plasma, with concentrations ranging from 3 to 30 μgml^{-1} and accounting for up to 0.05% of total plasma protein (343).

Adiponectin acts as an insulin-sensitizing hormone (344, 345) but also has anti-

inflammatory (346) and anti-atherogenic effects (347, 348). Unlike leptin and TNF- α , circulating adiponectin levels are decreased with increasing adiposity (349, 350). Low levels of adiponectin may thus provide a link between obesity and risk of colorectal cancer. This hypothesis has been evaluated in several epidemiological studies. Otake et al. performed a case-control study to examine visceral fat accumulation and the levels of adiponectin in Japanese patients with colorectal adenoma. They reported that the patients with colorectal adenoma showed significantly more visceral fat and significantly less plasma adiponectin concentration in comparison with the controls (odds ratio (OR)=2.19, 95% confidence interval (95% CI)=1.47-3.28; $p<0.001$ and OR=0.24, 95% CI=0.14-0.41; $p<0.001$, respectively) (351). Wei and coworkers conducted another study to evaluate the association between plasma adiponectin and risk of colorectal cancer among 18,225 men in the Health Professional Follow-up Study. In this ongoing prospective cohort study, they observed a statistically significant inverse association between plasma levels and risk of colon cancer (352). However, one observational case-control study contradicts the epidemiological data linking adiponectin to colon carcinogenesis (353). In that study, serum adiponectin levels were determined from 381 colorectal cancer cases and 381 controls. The results showed that there was no association between adiponectin level and risk of colorectal, colon, or rectal cancer in the whole study population. More recently, a proinflammatory role of adiponectin was suggested in chemically induced colitis by demonstration that adiponectin induced production of proinflammatory cytokines and inhibited bioactivity of protective growth factors (354). Furthermore,

in the human colonic epithelial cell line HT-29, adiponectin stimulated proliferation and production of proinflammatory cytokines, with a potentially distinct role of the globular fragment compared with full-length adiponectin (355).

The potential inhibitory role of adiponectin on tumorigenesis is also suggested by the demonstration that adiponectin was inversely correlated with fasting plasma insulin (356). Several studies have supported the association of elevated insulin production and decreasing levels of IGFBP-1 and IGFBP-3, with an increased risk of developing colorectal malignancies (357, 358). Therefore, low adiponectin levels can potentially lead to carcinogenesis through altered effect of insulin on tumor cell proliferation. Another mechanism linking adiponectin with reduced tumorigenesis is through signaling pathways. Several signaling molecules such as 5'-AMP-activated protein kinase (AMPK), nuclear factor κ B (NF- κ B), PPAR- α and p38 MAPK are known to mediate adiponectin-induced metabolic effects (358). More recently, JNK and STAT3 were also shown to be downstream effectors of adiponectin (359). Through these signaling pathways, adiponectin might reduce the risk for developing colorectal cancers. Finally, adiponectin may also inversely regulate angiogenesis through induction of apoptosis in vascular endothelial cells by activating the caspase cascade (360).

Interleukin (IL)-6 and CRC

Inflammation has been linked to risk of colorectal cancer (361). Excess adiposity is linked to higher serum concentrations of inflammatory markers, IL-6,

TNF- α and C-reactive protein (CRP) (362).

IL-6 is mainly produced by visceral fat (363) and the elevated IL-6 production induced by leptin promotes cell proliferation in an Apc (Min/+) colon epithelial cell line (364). In vivo, IL-6 binds to its soluble receptors (sIL-6R, sIL-11R, soluble ciliary neurotrophic factor receptor) and stimulates several types of target cells which could not been stimulated by IL-6 alone. This process has been named trans-signaling. Jones et al have shown that in several chronic inflammatory diseases, including colon cancer, trans-signaling via the sIL-6R complexed to IL-6 is a crucial point in the maintenance of the disease, by promoting transition from acute to chronic inflammation (365).

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PART 3

EXPERIMENTAL INVESTIGATIONS

1. Adiposity-Related Protection of Intestinal Tumorigenesis:
Interaction with Dietary Calcium

Accepted for publication in Nutrition and Cancer

1.1 Abstract

Although high calcium diets have been reported to reduce the risk of colorectal cancer, our preliminary data with the *Apc^{Min/+}* mouse shows a paradoxical increase in intestinal tumor loads (>65%) with high calcium diets. Since we previously demonstrated that increasing dietary calcium reduces adiposity, and *Apc^{Min/+}* mice on high calcium diets exhibited profound loss of adipose tissue, we hypothesized that loss of an adipose tissue-derived tumor suppressor factor(s) resulted in increased tumor susceptibility in animals on the high calcium diet. Accordingly, tumor prone *Apc^{Min/+}* mice were crossed with obesity prone *A^y/a* mice to generate obese *A^y/Apc^{Min/+}* mice. Low (0.2%), normal (0.5%), and high (1.2%) calcium diets were fed to both *A^y/Apc^{Min/+}* mice and *Apc^{Min/+}* mice from 35-40 days until 90 days of age (n=21/strain, n=7/ diet group). The high calcium diet reduced weight gain in both strains ($p < 0.01$) and reduced fat pad mass by 46%-57% in *A^y/Apc^{Min/+}* ($p < 0.004$) and by 65%-82% in *Apc^{Min/+}* ($p < 0.03$). *Apc^{Min/+}* mice on the high calcium diet exhibited an increase in tumor number (76 vs. 29, $p = 0.009$), but this effect was not seen in the *A^y/Apc^{Min/+}* mice. β -catenin and cyclin D1 gene expression were significantly induced with high calcium diet in intestinal tumor tissue of *Apc^{Min/+}* mice but not in *A^y/Apc^{Min/+}* mice. We conclude that the differential effect of dietary calcium on intestinal tumorigenesis in lean vs. obese *Apc^{Min/+}* may result from the loss of adipose-derived protective factor(s) due to the substantial loss of body fat in *Apc^{Min/+}* mice fed a high calcium dairy diet, increasing β -catenin and cyclin D1 in tumors.

1.2 Introduction

Epidemiological studies and clinical trials have reported an inverse association between dietary calcium intake and the incidence of colorectal cancer. As early as 1985, Lipkin and Newmark published data indicating that dietary calcium was inversely associated with epithelial proliferation (1). Recent analysis of dietary patterns strongly suggests that high consumption of dairy products and fruits and vegetables may be associated with a decreased risk of colorectal adenomas (2). Clinical trials further indicate that supplementary calcium significantly reduces adenoma recurrence (3) and the risk for all types of colorectal polyps (4). Studies in rodents also have shown that a high calcium intake causes a reduction of epithelial cell proliferation in the colon and increases apoptosis in the distal colonic epithelium (5). However, some epidemiologic studies have yielded conflicting findings (6, 7).

The C57BL/6J-*Apc*^{Min} mouse is highly susceptible to spontaneous intestinal adenoma formation due to a heterozygous, dominant mutation in the *Apc* (adenomatous polyposis coli) gene. The normal *APC* gene functions as a tumor suppressor and is involved in the up-regulation of genes implicated in normal differentiation and down-regulation of β -catenin transcriptional activity and transcriptional targets such as cyclin D1 (8). Loss of APC function with germ-line and/or spontaneous mutations of the *APC* gene initiates tumorigenesis in familial adenomatous polyposis and sporadic colorectal cancer (9, 10). The *Apc*^{Min/+} mouse model mimics the rapid development of adenomatous polyps that occur in humans with *APC* gene mutations, although tumors in this model occur

predominantly in the small intestine (11). Previous studies showed that colonic epithelial cell proliferation of *Apc1638* mice (similar to *Apc^{Min/+}* tumor model) was significantly increased after consumption of a diet very low in calcium and vitamin D, and that normalizing dietary calcium and vitamin D could prevent these changes (12). However, Harris and coworkers (13) did not find an effect of a high calcium diet on polyp number or tumor load over the entire intestine in female *Apc^{Min/+}* mice. In contrast, our preliminary observations suggested that dairy calcium intake paradoxically promoted intestinal tumor growth in *Apc^{Min/+}* mice, and that this effect was accompanied by a profound loss of adipose tissue. Since we have previously demonstrated that increasing dietary calcium effectively reduces adiposity (14, 15), we hypothesized that this excessive loss of adipose tissue resulted in increased tumor susceptibility in *Apc^{Min/+}* mice on the high calcium dairy diet.

Agouti mutations result in ectopic expression of the agouti protein in mice. Lethal yellow (*A^y*) mutation is one of the dominant agouti mutations. It is characterized by embryonic lethality of the homozygous *A^y/A^y* genotype (16). The *A^y* genotype results from a large deletion that includes the coding regions of the *Raly* (heterogeneous nuclear ribonucleo-protein associated with lethal yellow) gene. Consequently, the *A^y* allele is under the control of the *raly* promoter, and ectopic overexpression of the agouti protein produces the effects, including yellow coat color, obesity, diabetes and tumor susceptibility (16, 17).

The current study was undertaken to examine the intestinal tumor load, in tumor-prone mice with varying degrees of adiposity provided diets containing

various levels of calcium. We report that the loss of marginal adipose stores in $TAp^{Min/+}$ mice fed the high calcium dairy diet was associated with elevated tumor load, while this was not the case in $A^y/Apc^{Min/+}$ mice with greater remaining adipose tissue. Our results provide evidence that there is a critical level of adipose tissue required to maintain a protective effect against intestinal tumorigenesis.

1.3 Materials and methods

Mice breeding and genotyping

The experiment was approved by the University of Tennessee Institutional Animal Care and Use Committee. To produce obese tumor-prone mice, we bred male C57BL/6J- $Apc^{Min/+}$ (Jackson Laboratory, Bar Harbor, ME) at 6-7 weeks of age and female C57BL/6J- A^y mice to generate $A^y/Apc^{Min/+}$ mice; our preliminary data demonstrated that this cross had no effect on the intestinal tumor load. Mice were genotyped for the Min mutation using DNA obtained by the HotSHOT (Hot sodium Hydroxide and Tris) extraction protocol (18) and a protocol provided by the Jackson Laboratory (19), and by coat color, for agouti.

Experimental Design

$A^y/Apc^{Min/+}$ and $Apc^{Min/+}$ mice (n=21/strain) were generated from our breeding colony and genotyped as described above. Mice (by strain) were randomly divided into 3 dietary groups (7 animals per group) with three levels of calcium (0.2%, 0.5% or 1.2% w/w) either in the form of carbonate calcium (0.2% and 0.5%) or non-fat dried milk (1.2%, high calcium dairy diet). Protein content in all

diets was adjusted to 14 energy %. The mid-range (normal or standard) diet was based on content in the AIN-93G diet (i.e. 0.5% calcium diet). Each dietary group consisted of 4-5 males and 2-3 females. During the experiment, the mice were housed individually and had free access to food and water. Food intake was measured daily and animals were weighed twice per week.

At 90 days of age, all mice were anesthetized with intraperitoneal injections of sodium pentobarbitol (50 mg/kg body weight). Plasma samples were collected via cardiac puncture and kept at -20 °C. To determine the number and size of intestinal tumors, the entire intestinal tract was removed, flushed with ice cold phosphate-buffered saline (PBS) and opened longitudinally as described previously (20). Tumor number and size were determined using a dissecting microscope (18X) (20). Four fat depots (abdominal, subcutaneous, epididymal and perirenal), and skeletal muscle (soleus and gastrocnemius) mass were weighted and immediately frozen at -80°C, with pooled samples of small intestinal tumors from individual mice.

Plasma leptin and insulin radioimmunoassays

Leptin and insulin plasma concentrations were measured using radioimmunoassay kits for mouse leptin and rat insulin, respectively (Linco Research Inc., St. Charles, MO).

Total RNA extraction and quantitative real-time PCR

Total RNA from mouse intestine tissue was extracted by using a total cellular RNA isolation Kit (Ambion, Inc, Austin, TX) according to the manufacture's instruction. Mouse Bax gene, Bcl-2 gene, β -catenin gene and cyclin D1 gene expression levels of tumor intestine tissues were measured quantitatively using 7300 Real Time PCR system (Applied Biosystems, Foster City, CA) with One-step RT-PCR Master Mix (Applied Biosystems, Foster City, CA). The mRNA quantitation for each sample was normalized to 18s. The primers and probes were ordered from Applied Biosystems (Foster City, CA).

Statistical analysis

All data were expressed as mean $\pm$ SE. Data were evaluated for statistical significance by one-way and two-way analysis of variance (diet $\times$ strain), and significantly different group means were then separated by the least significant difference test by using SPSS 12.0 (SPSS Inc. Chicago, IL).

1.4 Results

Dietary consumption and body weight gain

Dietary calcium had no effect on food intake of each strain of mice (Table 1); however, reduced body weight gain was observed in both $A^y/Apc^{Min/+}$ and $Apc^{Min/+}$ mice fed medium and high levels of calcium (Table 2) compared to the low calcium diet group. The high calcium dairy diet reduced weight gain by 64% and 98% in $A^y/Apc^{Min/+}$ and $Apc^{Min/+}$, respectively.

Table 1: Average food consumption (g/day) of $A^y/Apc^{Min/+}$ and $Apc^{Min/+}$ mice^a

Diet	$A^y/Apc^{Min/+}$ mice	$Apc^{Min/+}$ mice
low calcium	4.00±0.11	3.47±0.10
medium calcium	3.90±0.10	3.03±0.12
high calcium dairy	4.16±0.17	3.28±0.24

^a Data expressed as mean ± SE.

Table 2: Body weight gain (g) (in 5-6 weeks) of $A^y/Apc^{Min/+}$ and $Apc^{Min/+}$ mice in different diet groups^a

Diet	$A^y/Apc^{Min/+}$ mice	$Apc^{Min/+}$ mice
low calcium diet	13.38±1.51	2.64±0.85
medium calcium diet	8.78±1.72	1.95±0.74
high calcium dairy diet	4.89±1.76*	0.03±0.95

^a Data expressed as mean ± SE.

* $p < 0.01$ vs. low calcium diet group in $A^y/Apc^{Min/+}$ mice

Effects of different calcium diets on fat pad mass and skeletal muscles

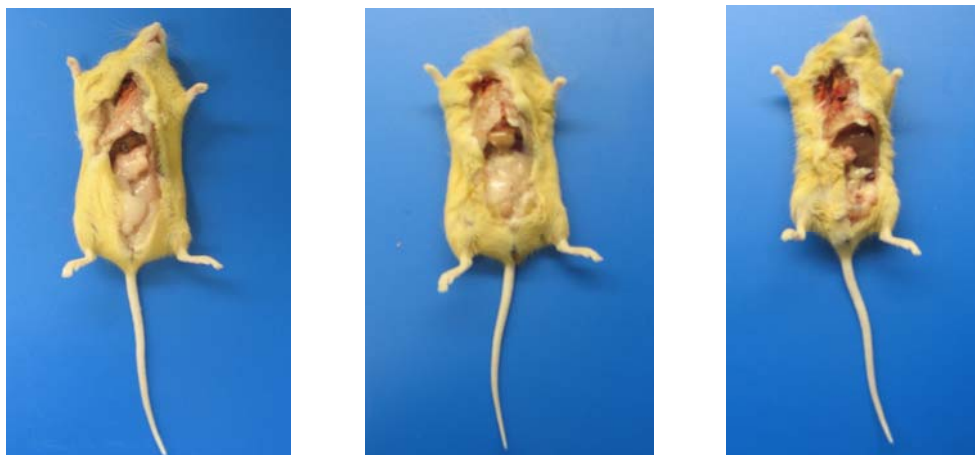
The high calcium diets resulted in marked reduction in body fat in both strains of mice ($p = 0.008$ in $A^y/Apc^{Min/+}$; $p = 0.023$ in $Apc^{Min/+}$). Feeding the $A^y/Apc^{Min/+}$ mice the high calcium dairy diet resulted in reductions in abdominal, perirenal and subcutaneous fat depots of 47%, 58% and 53%, respectively ($p = 0.003$, Fig.3, Table 3) compared to the low calcium diet. This decrease was also found in the $Apc^{Min/+}$ mice, in which the high calcium dairy diet caused decreases in three fat mass of 68%, 82% and 71%, respectively (Table 3).

Diet had no effect on skeletal muscle (soleus and gastrocnemius) mass (Table 3).

Effects of different calcium diets on tumor number and size

The level of dietary calcium had no effect on tumor multiplicity in the $A^y/Apc^{Min/+}$ mice (Table 4). A lack of effect was also observed in $Apc^{Min/+}$ mice fed the low and medium levels of calcium. However, when $Apc^{Min/+}$ mice were placed on a high calcium diet, their tumor number was significantly higher by 74% and 150% than that observed in normal and low calcium groups ($p < 0.009$), respectively.

A



B

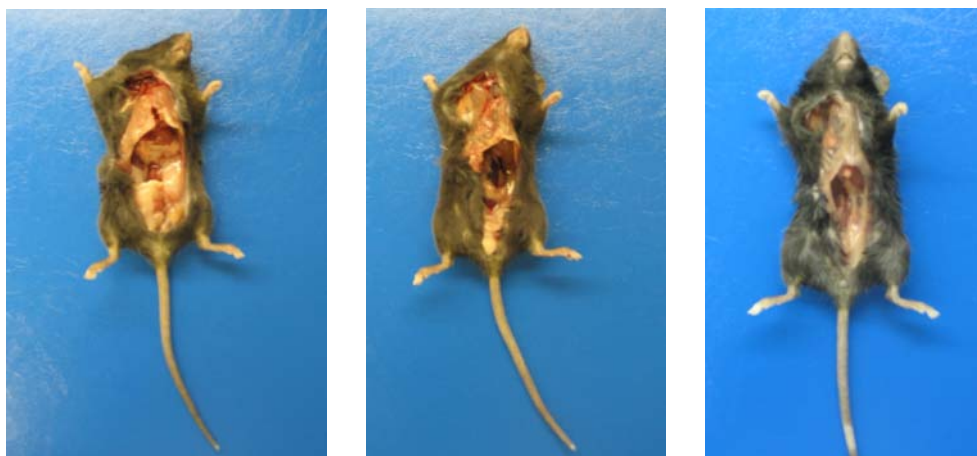


Fig. 3 Representative photographs of gross change in mice fed low (left), medium (middle) and high (right) calcium diets.

A: $A^y/Apc^{Min/+}$ mice; **B:** $Apc^{Min/+}$ mice.

Table 3: Effects of different calcium diets on fat mass and skeletal muscles[†]

	<i>A^y/Apc^{Min/+}</i> mice			<i>Apc^{Min/+}</i> mice		
	0.2% calcium	0.5% calcium	1.2% calcium	0.2% calcium	0.5% calcium	1.2% calcium
	(n=7)	(n=7)	(n=7)	(n=7)	(n=7)	(n=7)
Abdominal	1.92±0.19 ^a	1.47±0.23 ^a	1.03±0.28 ^b	0.40±0.1*	0.32±0.07*	0.13±0.03**
Perirenal	0.92±0.11 ^a	0.63±0.09 ^a	0.39±0.07 ^b	0.17±0.06	0.10±0.03	0.03±0.01
Epididymal	0.16±0.02	0.16±0.04	0.14±0.04	0.65±0.01	0.76±0.01	0.053±0.01
Subcutaneous	2.93±0.24 ^a	1.96±0.26 ^a	1.38±0.32 ^b	0.48±0.12*	0.38±0.09*	0.14±0.03**
Total fat mass	5.86±0.51 ^a	4.18±0.58 ^a	2.87±0.6 ^b	1.09±0.28*	0.85±0.19*	0.36±0.06**
Soleus	0.01±0.004	0.01±0.005	0.01±0.005	0.02±0.01	0.02±0.01	0.02±0.003
Gastrocnemius	0.05±0.01	0.06±0.01	0.06±0.01	0.06±0.01	0.06±0.01	0.08±0.01

[†]Data expressed as mean ± SE

^{a,b} Values in each row with different superscript letters are significantly different by One-way ANOVA ($p<0.05$)

* Value is significantly different from ** value by One-way ANOVA ($p<0.05$)

Table 4. Effects of different calcium diets on tumor in the colon and intestine of *A^y/Apc^{Min/+}* mice and *Apc^{Min/+}* mice

	<i>A^y/Apc^{Min/+}</i> mice			<i>Apc^{Min/+}</i> mice		
	0.2%	0.5%	1.2%	0.2%	0.5%	1.2%
	calcium	calcium	calcium	calcium	calcium	calcium
	(n=7)	(n=7)	(n=7)	(n=7)	(n=7)	(n=7)
Total tumor number/group						
Colon						
< 1mm	- ^a	-	-	-	-	-
1.01-2mm	1 ^b	1	1	1	-	2
2.01-3mm	1	1	1	-	1	3
3.01-4mm	-	-	1	2	1	2
>4mm	-	-	-	-	1	-
Small intestine						
<0.5mm	17	13	9	8	5	4
0.51-1mm	105	111	108	68	107	137
1.01-1.50mm	70	109	104	84	125	258
1.51-2mm	27	49	60	27	54	105
2.01-2.5mm	6	15	14	15	12	21
2.51-3mm	4	2	2	5	10	4
>3mm	-	-	-	1	2	1
Total number/group	231	301	300	211	318	537
Average tumor/mouse ^c	33.43±2.65	42.86±8.66	47.71±2.55	29.71±5.13 ^d	42.57±6.90 ^d	74.29±12.80 ^e
Average tumor size	1.21±0.45	1.31±0.48	1.33±0.35	1.32±0.55	1.41±0.88	1.42±0.48

^a None observed

^b number of tumors

^c Data expressed as mean ± SE

Correlation between body fat mass and tumor number

There were significant negative correlations between fat pad mass (sum of abdominal, subcutaneous, epididymal and perirenal fat) and tumor number in both mouse strains ($r = -0.673$ in $A^y/Apc^{Min/+}$ mice, $p < 0.001$; $r = -0.450$ in $Apc^{Min/+}$ mice, $p < 0.002$) (Fig.4).

Effects of calcium diets on plasma leptin and insulin levels

Plasma leptin levels in the $A^y/Apc^{Min/+}$ mice were progressively lower in the medium and high calcium groups compared to the low calcium group (28.2 ± 9.1 ng/ml and 19.0 ± 2.0 ng/ml versus 51.4 ± 7.6 ng/ml, respectively), but diet had no effect on serum leptin levels in the $Apc^{Min/+}$ mice (Fig.5). Two-way ANOVA analysis showed that the overall plasma leptin levels were higher in the $A^y/Apc^{Min/+}$ mice as compared to $Apc^{Min/+}$ mice with all levels of dietary calcium ($p < 0.01$).

Dietary calcium exerted no effect on plasma insulin levels in either $A^y/Apc^{Min/+}$ or $Apc^{Min/+}$ mice (Fig.6); however, the overall insulin levels of $Apc^{Min/+}$ mice were higher than that of $A^y/Apc^{Min/+}$ mice at each level of calcium diet ($p < 0.001$).

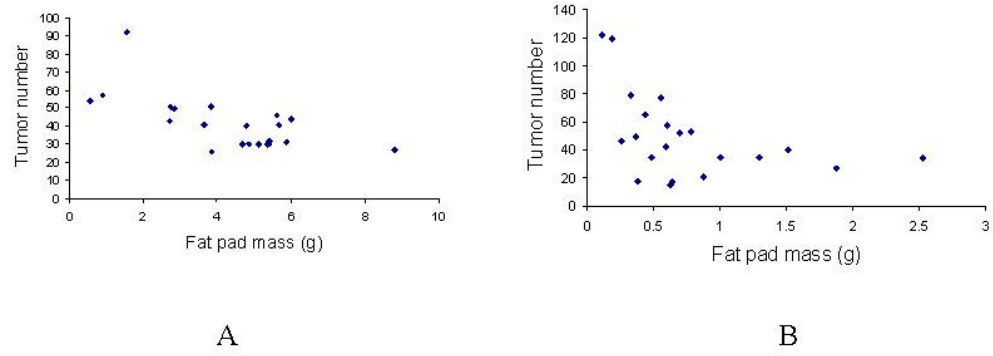


Fig.4 Correlation between body fat mass and tumors

A: $A^y/Apc^{Min/+}$ mice; B: $Apc^{Min/+}$ mice

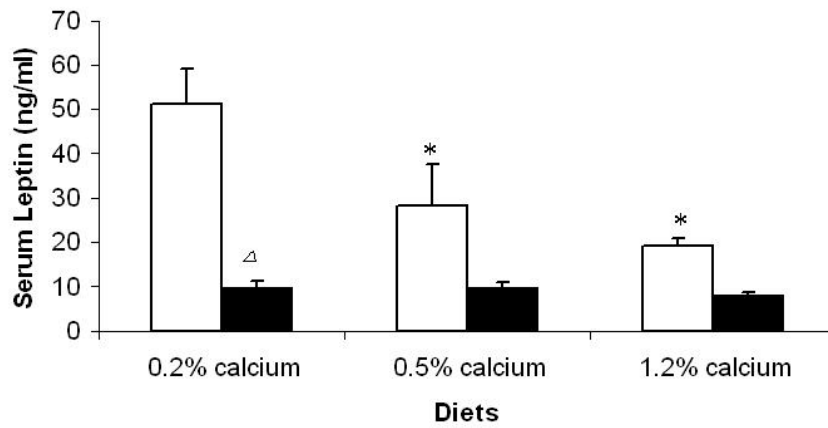


Fig.5 Effects of calcium diets on plasma leptin levels of $A^y/Apc^{Min/+}$ (□) mice and $Apc^{Min/+}$ mice (■).

* $p < 0.02$ vs. low calcium diet group in $A^y/Apc^{Min/+}$ mice;

^Δ $p < 0.001$ vs. $A^y/Apc^{Min/+}$ mice within low calcium diet group.

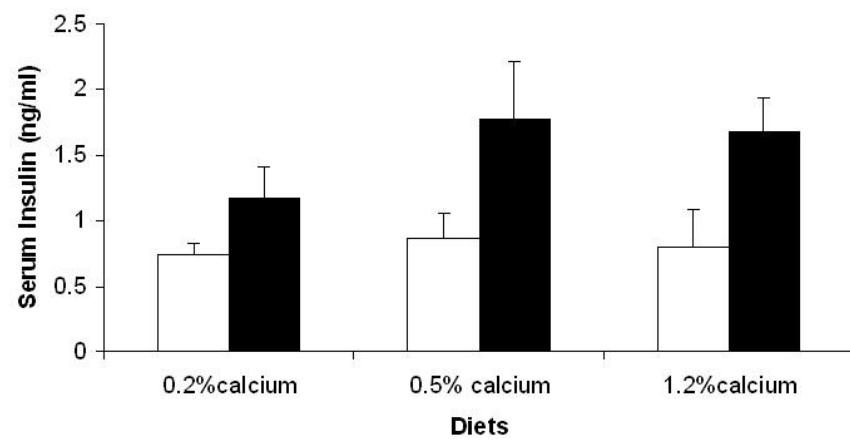


Fig.6 Effects of calcium diets on plasma insulin levels of

$A^y/Apc^{Min/+}$ (□) mice and $Apc^{Min/+}$ mice (■).

Effects of calcium diets on intestinal tumor β -catenin and cyclin D1 gene expression

In $A^y/Apc^{Min/+}$ mice, intestinal tumor there were no significant differences in β -catenin levels between the high and low calcium diets.

In contrast, $Apc^{Min/+}$ mice on the high calcium diet had tumors with 142%^{*} higher levels of β -catenin gene expression (Fig. 8D, $p < 0.05$) in comparison with the low calcium diet.

In addition cyclin D1 mRNA, a transcriptional target of β -catenin, was also significantly higher in the mice fed the high calcium dairy diet compared to those fed the low and medium calcium diet groups (Fig. 9).

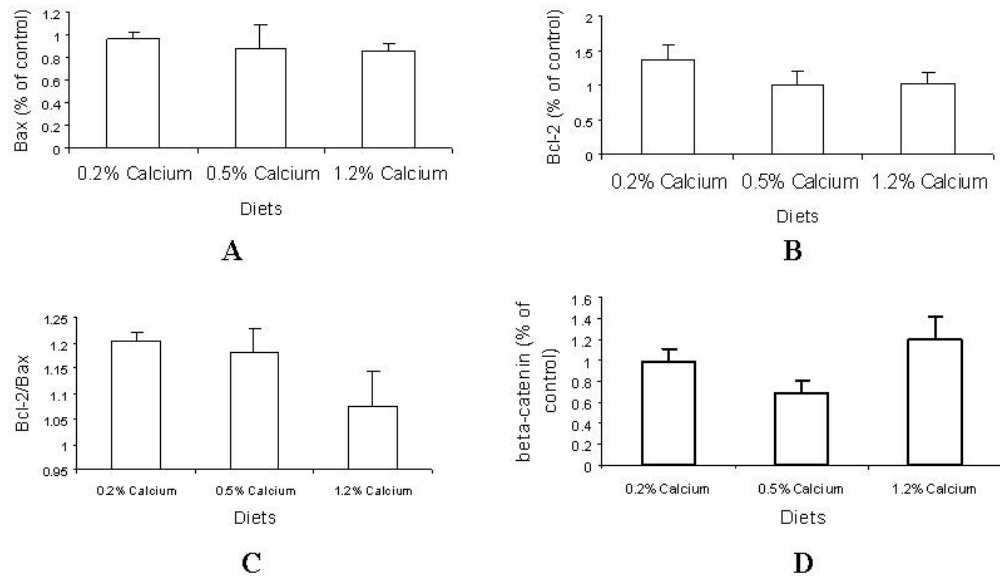


Fig.7 Effects of calcium diets on $A^y/Apc^{Min/+}$ mice intestinal tumor tissue gene expression. **A:** Bax; **B:** Bcl-2; **C:** Bcl-2/Bax ratio; **D:** β -catenin

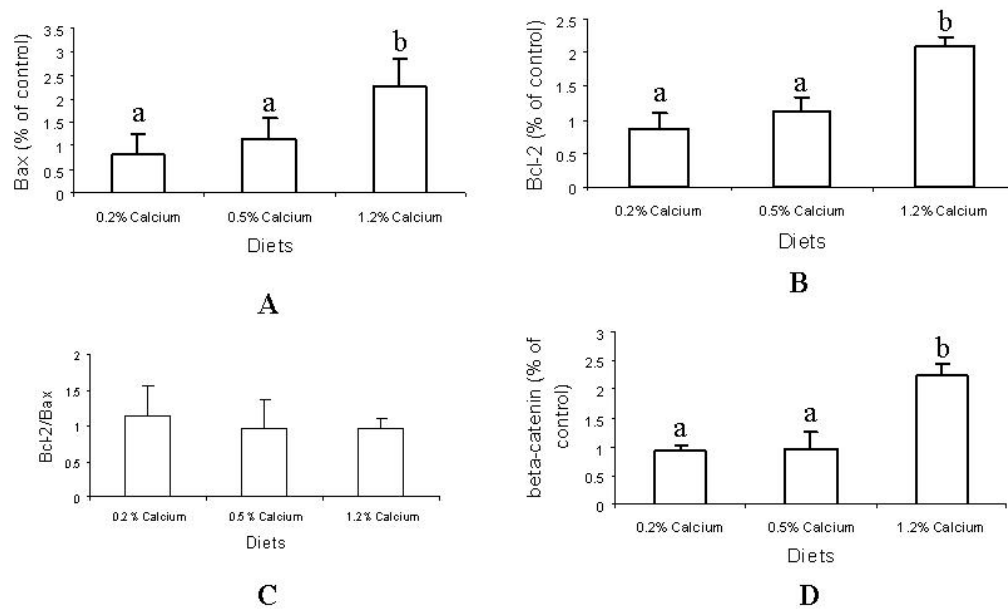


Fig.8 Effects of calcium diets on *Apc*^{Min/+} mice intestinal tumor tissue gene expression.

A: Bax; **B:** Bcl-2; **C:** Bcl-2/Bax ratio; **D:** β -catenin

Means without a common letter differ by one-way ANOVA, $p < 0.05$

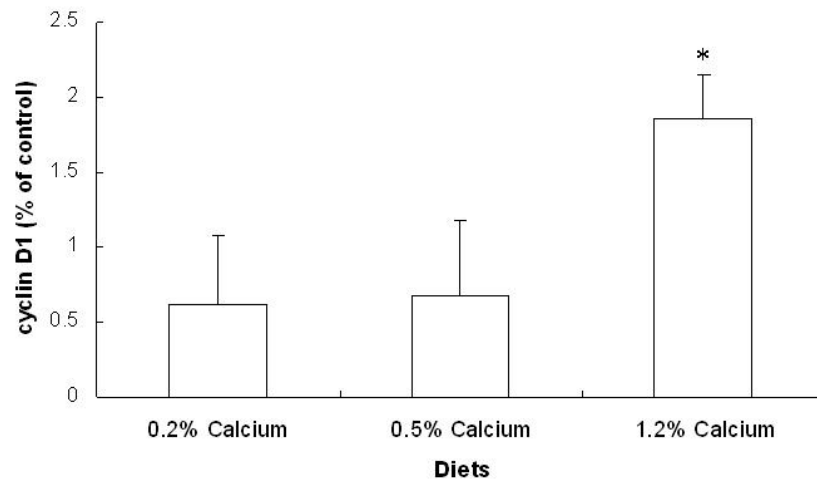


Fig.9 Effects of calcium diets on *Apc*^{Min/+} mice intestinal tumor cyclin D1 gene expression.

* $p < 0.05$ vs. low and medium calcium diet group

1.5 Discussion

Colorectal cancer is the third most commonly diagnosed type of tumor in the United States and the second or third most common cause of cancer-related mortality (21). Its prevalence is higher in Western societies and environmental factors, in particular diet, is believed to have a significant impact on this disease process. Epidemiological studies suggest an inverse relationship exists between dietary calcium and colorectal cancer risk (22). Calcium may help to directly inhibit colonic epithelial proliferation by precipitating bile acids and fatty acids and thereby reduce the cellular toxicity within the colonic lumen (23). Extracellular calcium may also act in the regulation of cell proliferation and differentiation by sensing CaSR (calcium sensing receptors), followed by a cascade of diverse intracellular signaling pathways (24). The inverse effect between dietary calcium and colon cancer risk has also been linked mechanistically to indirect effects on vitamin D metabolism (i.e., changes in circulating calcitriol and 25-(OH)-D₃), effects as a first messenger (i.e., via calcium-sensing receptor) and as a second messenger (changes in the intracellular calcium concentrations), with subsequent downstream effects on pathways influencing the balance between proliferation and apoptosis (25). However, studies evaluating the effects of dietary calcium on tumorigenesis using experimental rodent models have been equivocal and this paper explores a novel mechanism which appears to potentially contribute to this variability.

In contrast to data describing an inverse relationship between dietary calcium and colon cancer risk, recent data from the Women's Health Initiative

demonstrate no effect of moderately long-term (7 year) calcium supplementation on the incidence of colorectal cancer among post-menopausal women (26). This negative finding may be attributable, in part, to the long latency associated with colorectal cancer. However, in light of findings from the present study, it is also possible that putative protective effects of calcium against colorectal cancer may be least apparent among lean individuals, suggesting that future studies of calcium and colorectal cancer should consider stratification of subjects according to adiposity.

A diet based on AIN-93G (Research Diets, Inc. NJ), a dietary formula for gestating and growing rodents as recommended by the American Society for Nutrition (27) was modified with three levels of dietary calcium. The typical AIN-93G diet contains 0.5% calcium by weight, or 1.2mg/kcal. If one assumes an equivalency between recommended levels in laboratory animals and humans, the 0.5% calcium diet used in this experiment would be equivalent to a human intake of 1,000 mg, while the low (0.2%) and high (1.2%) calcium diets would be equivalent to 400 mg and 2,400 mg for humans, respectively. These equivalencies cannot be calculated with certainty, as establishing human equivalency with experimental rodent diets is problematic; several approaches have been suggested, including body weight, body surface area, metabolic rate and energy density (28). However, none of these produce a satisfactory equivalency for calcium. For example, using the energy density approach demonstrates that the 0.5% level of calcium used in The AIN-93 diet (27) and recommended by the National Research Council (US) (29) contains 1.2 mg Ca/kcal, which extrapolates to a

human dietary calcium intake of 3,000 mg/day for an individual consuming 2,500 kcal/day, a level in excess of the Upper Limit for safety established by the Food and Nutrition Board of the National Academy of Sciences (US). This extrapolation is clearly not tenable, as this level of calcium intake (0.5%) is not toxic to rats or mice and is instead consistent with a level that supports optimal performance (29). Moreover, Chandler and Cragle (30) demonstrated that optimal growth and skeletal mineralization performance of the growing laboratory rat occurred between 0.5 and 0.67% calcium, consistent with the aforementioned recommendations (27, 29). Consequently, in the absence of any ideal method of calculating mouse-human equivalency, in the present study we have evaluated departures from the recommended levels for the mice, bracketing the recommended level with a lower level (0.2%, or 40% of recommended levels) and a higher level (1.2%, or 2.4-fold higher than recommended and 3-fold higher than the lowest level used in this study).

This study demonstrated that a high calcium diet surprisingly resulted in a pro-tumorigenic effect which may not be related to previously reported mechanisms of calcium, but rather indirectly associated with its anti-obesity effects. The amount of calcium and the vehicle of the supplementation appear to be critical for this response, particularly in *Apc*^{Min/+} mice, an accepted model of intestinal tumorigenesis which has been shown to be sensitive to dietary intervention (31-34). Previously, it had been demonstrated that providing *Apc*^{Min/+} mice variable levels of elemental calcium in the form of calcium carbonate (0.2% - 1.2%, w/w) had no effect on tumor load (34), and these results have been recapitulated in our

laboratory (unpublished data). Data from human studies indicate that elemental calcium and dairy calcium have responded equivalently (35). However, we previously found that when calcium was provided in the form of non-fat dry milk, tumor number was 52% higher in animals provided a high dose of calcium (1.2%, w/w) versus a low dose of calcium (0.2%, w/w calcium carbonate) (an average of 62 tumors/mouse versus 41 tumors/mouse, respectively, from replicate studies) (unpublished data). This increase in tumorigenesis is in apparent opposition to the effect of dairy calcium on colon cancer biomarkers (36). It is possible that this effect is unique for dairy calcium and/or dairy products containing calcium; however, this is highly unlikely since dairy contains significant levels of other bioactive components that have been shown to decrease the number of tumors in animal models, such as vitamin D (37), butyrate (38) and sphingomyelin (39). Increasing circulating levels of 25-(OH)-D₃ have been shown to have an inverse relationship on cancer risk (40) and reduce epithelial cell proliferation (41). Calcium supplementation has been shown to decrease 25-(OH)-D₃ levels in *Apc*^{Min/+} mice (35). However, tumor number in *Apc*^{Min/+} mice were unaffected by treatment with the active form of vitamin D (1, 25-(OH)₂-D₃) or a non-calcemic active synthetic analogue (42). In addition, while differences were modest and not statistically significant, tumor loads were higher in the mid-range than low level calcium (carbonate) diets and fat deposits were decreased.

One consistent aspect associated with all of our studies was the virtual absence of adipose tissue in the *Apc*^{Min/+} mice fed high levels of dairy calcium. *Apc*^{Min/+} mice tend to have smaller fat pad mass compared to genetically matched

C57BL/6J controls when fed ad libitum, but nevertheless, adipose tissue is always visibly present. However, placing the animals on a high dairy calcium diet resulted in additional loss of residual adipose tissue. These results are consistent with the anti-obesity effect of dietary calcium (14, 43-45); an effect which is substantially more prominent with dairy calcium than it is with elemental calcium (44). Thus, it is possible that the neoplastic effects of dairy calcium under these experimental conditions are related to the profound loss of fat mass.

To address this question and confirm our results, we wanted to either eliminate adipose tissue via an alternative method while maintaining caloric intake (thus mimicking our experimental conditions), or enhance the levels of fat in *Apc*^{Min/+} mice such that a sufficient amount of residual adipose tissue would be present after placing the mice on a high dairy calcium diet. With regards to the first point, it has already been established that reducing caloric intake by 40% reduces tumor number by >50% in this model (46), and there is no other dietary mechanism known to achieve similar effects on reducing adiposity outside of adding dairy calcium. Regarding the second point, we couldn't sufficiently "fatten up" the mice prior to placing them on a high dairy calcium diet because they have a relatively short life span (<120-140 days) and it was observed that these mice do not consume enough food (typically 2.5-3 g/d) (34) to generate excess adipose tissue necessary for this type of an experiment.

Therefore, to adequately address this problem, we crossed the "tumor-prone" *Apc*^{Min/+} mouse with the "obesity-prone" *A^y/a* mouse to generate a "fat" *A^y/Apc*^{Min/+} mouse cross. For this experiment to be successful, the cross would

have to have the same genetic background to eliminate the introduction of tumor modifying loci, have no impact on tumorigenesis, and yet have larger fat pad mass compared to $Apc^{Min/+}$ mice. All of these conditions were met with the cross: the backgrounds of the mice were genetically matched (C57BL/6J strains), the cross did not modify tumor load (preliminary data not presented), and the amount of adiposity was significantly higher in the $A^y/Apc^{Min/+}$ mice versus the $Apc^{Min/+}$ mice. Recapitulating our earlier results, tumor number was significantly higher in the $Apc^{Min/+}$ mice fed the high dairy calcium diet (1.2%) compared to the mice fed standard (0.5%) or insufficient amounts of calcium (0.2%). These mice were almost devoid of any visible adipose tissue. However, tumor load was not significantly different in $A^y/Apc^{Min/+}$ mice fed the high dairy calcium diet despite the same dose response (inverse relationship) between dietary calcium and level of adipose tissue.

These data suggest that a minimum amount or threshold level of adipose tissue may be required to significantly attenuate the augmented tumorigenic response observed in the $Apc^{Min/+}$ mice on the high calcium diets. If this were the case, the differential effect of dairy calcium on intestinal tumorigenesis may result from the loss of adipose-derived signaling molecules (i.e., adipokines) due to the substantial loss of body fat. It is possible that one or more of these adipokines (i.e., leptin, resistin, adiponectin, TNF- α , etc.) could directly or indirectly suppress tumorigenesis. In support of this concept, there were significant negative correlations between body fat mass and tumor number in both strains of mice, suggesting that intestinal tumor load could be predicted, in part, by the level of

body fat. There is, in fact, precedent for this concept. Cachexia is defined as a syndrome of progressive wasting characterized by significant loss of adipose tissue and lean body mass (47). Patients with malignancy-related cachexia experience prolonged survival with nutritional intervention, and this improved outcome is significantly correlated with increased body fat (48). Body fat is lost more rapidly than skeletal muscle in cancer patients suffering from cachexia, and the level of whole body fat significantly predicts survival, whereas lean tissue does not (49).

Data from the present study cannot be interpreted to suggest that excess adipose tissue mass is protective against colorectal cancer, as there is a well established positive relationship between obesity and colorectal cancer. Instead, these data suggest that excessive loss of adipose tissue results in loss of critical adipocyte-derived protective factors, although these factors have not yet been definitively identified.

The $A^y/Apc^{Min/+}$ mice were significantly heavier with larger fat pad mass than their $Apc^{Min/+}$ counterparts, and this difference was reflected in higher circulating levels of leptin, a biomarker of adipose tissue (Fig. 3). We also observed that insulin levels in $Apc^{Min/+}$ mice were higher than that of $A^y/Apc^{Min/+}$ mice, which suggests moderate insulin resistance in these animals. Although insulin is a growth factor, this effect does not explain the increase tumor number in $Apc^{Min/+}$ on the high dairy calcium diet.

β -catenin expression was enhanced in $Apc^{Min/+}$ mice on the high dairy calcium diet. Elevated level of β -catenin in the cytoplasm and nucleus is an important

characteristic and driving force of colorectal cancers; modifying aberrant β -catenin expression can influence tumorigenesis (50). One of the transcriptional targets of nuclear β -catenin is cyclin D1, which directly contributes to cell proliferation in these neoplastic cells. Cyclin D1 protein inactivates the retinoblastoma protein and promotes progression through the G1-S phase of the cell cycle (51). Consequently, higher cyclin D1 gene expression in the tumors of $Apc^{Min/+}$ mice on a high calcium dairy diet (Fig. 7) is consistent with increased tumor growth as well as higher levels of β -catenin, when compared to the low calcium fed group.

In summary, when $Apc^{Min/+}$ mice were fed a diet high in dairy calcium, their tumor frequency increased by 74% and 150% as compared to mice fed standard and suboptimal levels of calcium, respectively. Concomitantly, these animals experienced substantial loss of adipose tissue and, when a modicum of adipose tissue was retained in animals fed the high calcium dairy diet, as a result of cross-breeding of “tumor-prone” $Apc^{Min/+}$ mouse with “obesity-prone” A^y/a mouse to generate a “fat” $A^y/Apc^{Min/+}$ mouse, this enhanced tumorigenesis was abrogated. These data suggest that excessive loss of marginal adipose tissue stores may promote tumorigenesis, and the differential effect of dietary calcium on intestinal tumorigenesis may result at least in part, from the loss of adipose-derived protective factor(s) due to the absence of body fat. Studies are currently underway in our laboratories to identify the putative tumor-suppressor adipokine(s).

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**2. The Anti-proliferative Effect of Adiponectin on Human Caco-2
Colon Cancer Cells**

2.1 Abstract

Our previous work with *Apc*^{Min/+} (Min) mice suggests that loss of critical protective factor(s) from excessive loss of body fat on a high calcium diet results in increased intestinal tumor susceptibility. Given that obesity is a risk factor for colon cancer and that plasma adiponectin level is inversely associated with the incidence of obesity, we hypothesized that adiponectin may have anti-proliferative effect on Caco-2 cell proliferation, and that excessive loss of body fat in the already lean Min mice would lead to loss of this effect. Consequently, we have now examined the effect of human adipocytes and pre-adipocytes on growth of Caco-2 cells and the role of adiponectin in mediating this effect. Human adipocyte conditioned medium (HACM) inhibited Caco-2 cell growth by 28.0%-65.6% ($p < 0.03$) compared with DMEM, while human pre-adipocyte conditioned medium (HPCM) exerted no effect. Co-culturing Caco-2 cells with human adipocytes substantially suppressed Caco-2 proliferation by 62.8% ($\pm 10.1\%$, $p = 0.013$). Addition of a neutralizing anti-adiponectin antibody to the HACM and to the co-culture system completely reversed the inhibitory effects of human adipocytes on Caco-2 proliferation, indicating that adiponectin is responsible for this inhibitory effect. Similarly, a siRNA-mediated decrease in adiponectin protein in human adipocytes prevented the inhibitory effect of human adipocytes on Caco-2 proliferation. These data demonstrate that human adipocytes inhibit Caco-2 proliferation and that adiponectin is responsible for this effect. These results highlight the importance of stromal-cancer cell interactions

and suggest that adiponectin secreted by mature adipocytes may be a key regulator of colorectal cancer growth.

2.2 Introduction

Obesity is consistently associated with increased risk of colorectal cancer in men and women in both case-control and cohort studies (1). Among the biological mechanisms linking body size to colorectal cancer, mounting evidence suggests that adipokines such as leptin, adiponectin, and tumor necrosis factor- α (TNF- α), which are produced by adipocytes and function to regulate adipocyte homeostasis and metabolism, are associated with colorectal neoplasia (2).

Adiponectin is a cytokine secreted from white adipose tissue. Adiponectin acts as an insulin-sensitizing hormone (3, 4) but also has anti-inflammatory (5) and anti-atherogenic effects (6, 7). Unlike leptin and TNF- α , circulating adiponectin levels are decreased with increasing adiposity (8, 9). Low levels of adiponectin may thus provide a link between obesity and risk of colorectal cancer. This hypothesis has been evaluated in several epidemiological studies. Otake et al. performed a case-control study to examine visceral fat accumulation and the levels of adiponectin in Japanese patients with colorectal adenoma. They reported that the patients with colorectal adenoma showed significantly more visceral fat and significantly less plasma adiponectin concentration in comparison with the controls (odds ratio (OR)=2.19, 95% confidence interval (95% CI)=1.47-3.28; $p < 0.001$ and OR=0.24, 95% CI=0.14-0.41; $p < 0.001$, respectively) (10). Wei and coworkers conducted another study to evaluate the association between plasma adiponectin and risk of colorectal cancer among 18,225 men in the Health Professional Follow-up Study. In this ongoing prospective cohort study, they observed a statistically significant inverse association between plasma levels and

risk of colon cancer (11). However, one observational case-control study contradicts the epidemiological data linking adiponectin to colon carcinogenesis (12). In that study, serum adiponectin levels were determined from 381 colorectal cancer cases and 381 controls. The results showed that there was no association between adiponectin level and risk of colorectal, colon, or rectal cancer in the whole study population.

Our previous work with the *Apc^{Min/+}* mice suggests that loss of critical protective factor(s) from excessive loss of body fat on a high calcium diet results in increased intestinal tumor susceptibility (13), which is supported by the study of Abramowitz et al (14). Since plasma adiponectin is inversely associated with adiposity, it is possible that circulating levels of adiponectin partially explain the observed association between increased cancer susceptibility and either high or excessively low body fat mass. Therefore, we hypothesized that adiponectin may have anti-proliferative effects on colon cancer cell proliferation, and that excessive loss of body fat in the already lean *Min* mice would lead to loss of this effect. Consequently, we utilized an *in vitro* co-culture system and siRNA to examine the influence of human adipocytes on the growth of Caco-2 cells. We report here that the presence of adipocytes substantially suppressed Caco-2 proliferation, while a siRNA-mediated decrease in adiponectin protein in human adipocytes prevented the inhibitory effect of human adipocytes on Caco-2 proliferation. These data indicate that adiponectin may suppress colorectal tumorigenesis and that loss of this effect in lipodystrophic *min* mice may be responsible for observed increases in tumor load.

2.3 Materials and Methods

Reagents

Dulbecco's modified essential medium (DMEM), penicillin-streptomycin, Opti-MEM I reduced serum medium, Hanks' Balanced Salt Solution (HBSS) and 0.05% trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) and Eagle's minimum essential medium (MEM) were purchased from American Type Culture Collection (ATCC) (Manassas, VA). Adiponectin antibody was purchased from Sigma Chemical Co. (St. Louis, MO). Cell proliferation Biotrak ELISA system was obtained from GE Healthcare (Piscataway, NJ). Human adiponectin ELISA Kit was purchased from Linco Research (St. Charles, MO). CyQuant cell proliferation Kit was obtained from Invitrogen (Carlsbad, CA).

Cell culture

Human preadipocytes were maintained in preadipocyte medium. 12-well plate human adipocytes were purchased from Zen-Bio, Inc. (Research Triangle Park, NC) and cultured in adipocytes medium. Caco-2 cells were obtained from ATCC and maintained in MEM containing 2mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1mM non-essential amino acids, 50 IU/ml penicillin, 50 µg/ml streptomycin and 10% FBS.

Preparation of human adipocyte conditioned medium (HACM) and human pre-adipocyte conditioned medium (HPCM)

Human adipocytes and pre-adipocytes were maintained in adipocyte medium or pre-adipocyte medium for 24 h, respectively and conditioned medium was then prepared by incubation of the cells in serum-free medium (0.2% FBS containing DMEM) for 3 days. Control medium was prepared according to this procedure but without cells. The collected conditioned media were centrifuged at 800×g for 5 min to remove cellular components and used in fresh.

Cell proliferation experiments

Caco-2 cells were plated at a density of 1×10^4 cells/ml in 96-well plates and incubated at 37°C in DMEM plus 10% FBS. After 24 h the cultures were washed and refed with fresh HACM or HPCM. Control cells were cultured in serum-free DMEM. At 24, 48, 72, and 96 h the cells were washed with HBSS and cell proliferation was examined by immunohistochemistry for bromodeoxyuridine (BrdU) using the cell proliferation ELISA kit.

Transwell co-culture experiments

Human adipocytes or human preadipocytes were co-cultured with Caco-2 cells in the Transwell system separated by a polyester permeable support (Corning-Costar, Cambridge, MA). To examine the effect of adipocytes and preadipocytes on Caco-2 growth, Caco-2 cells were seeded on 12-well Transwell inserts (0.4 µm pore size). After 24 h culture in DMEM/10% FBS, cells were washed with HBSS

and the Transwells containing Caco-2 cells were transferred to 12-well plates with human adipocytes or preadipocytes previously maintained in serum-free DMEM for 24 h. Control cells were also grown on the Transwell inserts but in the absence of underlying cells. Media were changed every 2 days. Following the co-culture for 1, 2, 3 or 4 days, Caco-2 cells in the inserts were detached by 0.05% trypsin-EDTA and counted by hemocytometer. DNA content measurement was determined by using CyQuant Kit.

Human adiponectin neutralizing antibody treatment studies

Anti-adiponectin was added into the 3-days collected HACM or co-culture system at 150ng/ml before the experiment. The HACM treatment and co-culture experiment were then performed as described.

ELISA for human adiponectin

Human adiponectin was determined by using ELISA kit according to the manufacturer's protocol (Linco Research INC. St. Charles, MI).

siRNA

All siRNA materials were from Ambion (Austin, TX). KDalertTM GAPDH assay kit and GAPDH mRNA expression through qRT-PCR were used to monitor siRNA transfection in human adipocytes and to optimize the transfection protocol. Negative siRNA refers to SilencerTM Negative Control#1 siRNA.

Adiponectin siRNA refers to pre-designed siRNA ID 148359. siRNA stock solution was prepared in 20 μ M in RNase-free water.

RNA interference and transfection

siRNA transfection was performed on human adipocytes following maintaining in adipocyte medium for 24 h. Adipocytes were washed with Opti-MEM I reduced serum medium and maintained in 400 μ l per well prior to siRNA transfection. Cells were transfected with 2 μ l adiponectin siRNA or negative control using 3 μ l per well siPORTTM Lipid Transfection Agent according to the manufacturer's protocol. Four hours after addition of the siRNA/transfection agent complex, 3 ml adipocyte medium was added to each well. Twenty-four hours after transfection, adipocytes were washed with Opti-MEM I reduced serum medium and the transfection was repeated. Forty-eight hours after the second transfection, cell culture medium was replaced with serum-free medium. Then, the adipocytes were cultured for 24 h before the start of co-culture experiment. During the transfection and co-culturing, the down-regulation of the adiponectin targeted by siRNA was confirmed by analysis of its expression level using human adiponectin ELISA and quantitative RT-PCR.

Total RNA extraction and quantitative real-time PCR

Total RNA was extracted from human adipocytes transfected with adiponectin siRNA or negative controls by using total cellular RNA isolation Kit from Ambion (Austin, TX) according to the manufacture's instruction. RNA

concentration and quality were determined using Nanodrop ND-1000 Spectrophotometer (Nanodrop technologies, Wilmington, DE). RNA samples were stored at -80°C . Human adiponectin gene expression level was measured quantitatively using 7300 Real Time PCR system with One-step RT-PCR Master Mix (Applied Biosystems, Foster City, CA). The mRNA quantitation for each sample was further normalized by GAPDH. The primers and probes were obtained from Applied Biosystems.

Statistics analysis

All data were evaluated for normality of distribution and equality of variance prior to statistical analysis. Outcomes were shown as means $\pm$ SD and were evaluated for statistical significance by one-way analysis of variance (ANOVA) and *t* test to compare group means using SPSS 14.0 (SPSS Inc, Chicago, IL).

2.4 Results

HACM inhibits Caco-2 cell proliferation

Conditioned medium from human adipocytes inhibited Caco-2 cell proliferation. 25% HACM significantly inhibited Caco-2 cell proliferation at day 1 ($p = 0.000$) and day 3 ($p = 0.005$) (Fig. 10).

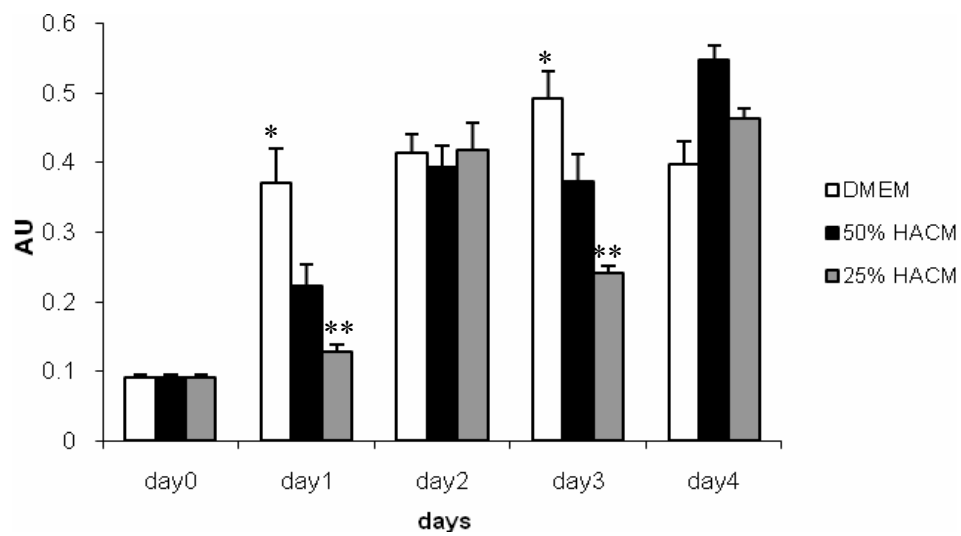


Fig. 10 Effects of HACM on Caco-2 cell proliferation
 * Value is significantly different from ** value by One-way ANOVA ($p < 0.05$)

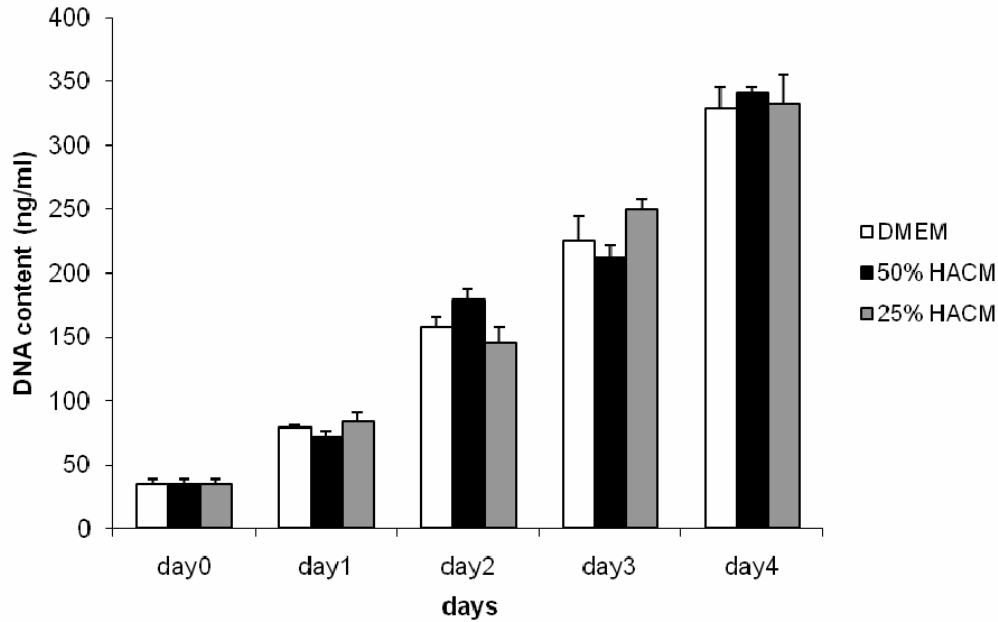


Fig. 11 Effects of HPCM on Caco-2 cell proliferation

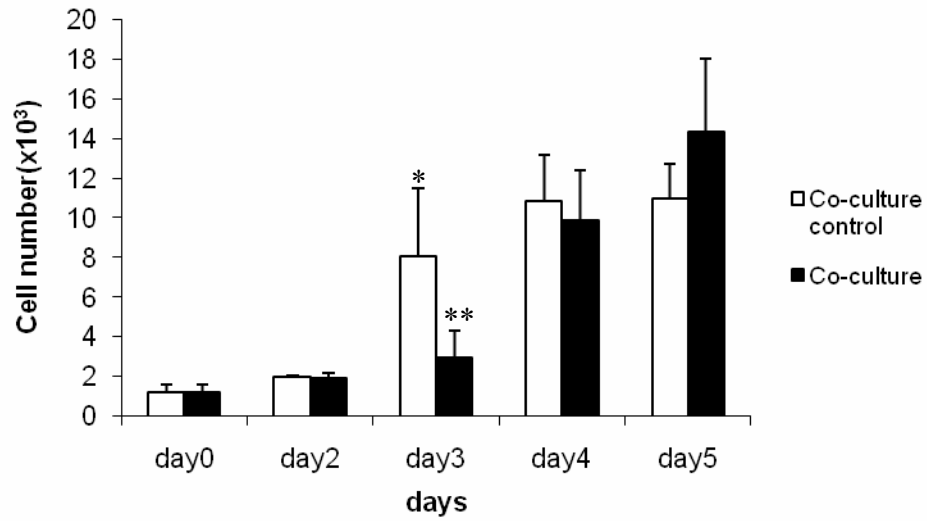
HPCM has no effect on Caco-2 cell proliferation

Compared with the DMEM treatment group, 3-days collected conditioned medium from human pre-adipocytes exhibited no effect on Caco-2 cell proliferation (Fig. 11).

Effects of human adipocyte and Caco-2 co-culture

Co-culturing human adipocytes and Caco-2 cells decreased Caco-2 cell proliferation, especially in day3 ($p < 0.05$, Fig. 12). Cell number was decreased by $62.8\% \pm 10.1\%$ ($p = 0.013$, Fig. 12A) compared to the control group, and DNA content level decreased by $32.2\% \pm 13.6\%$ ($p = 0.014$, Fig. 12B).

A



B

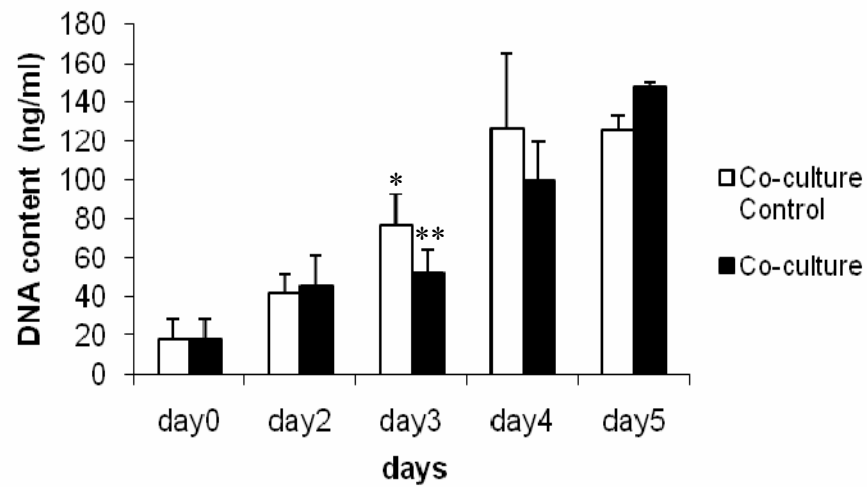


Fig. 12 Effects of co-culturing with human adipocytes on Caco-2 cell proliferation

A: cell number was counted by hemocytometer

B: DNA content was determined by CyQuant cell proliferation Kit.

* Value is significantly different from ** value by One-way ANOVA ($p < 0.05$)

Effects of human pre-adipocyte and Caco-2 cell co-culture

In contrast to the effects of adipocytes, co-culturing human pre-adipocytes and Caco-2 cells exerted no effect on Caco-2 cell proliferation (Fig. 13).

Effect of Anti-adiponectin

When the anti-human adiponectin-neutralizing antibody was added into the HACM, the growth-inhibiting effect of HACM was abolished (Fig. 14A), and there was an increase in Caco-2 cell proliferation on day2 (Fig. 14A). Similar results were found in the co-culture system (Fig. 14B).

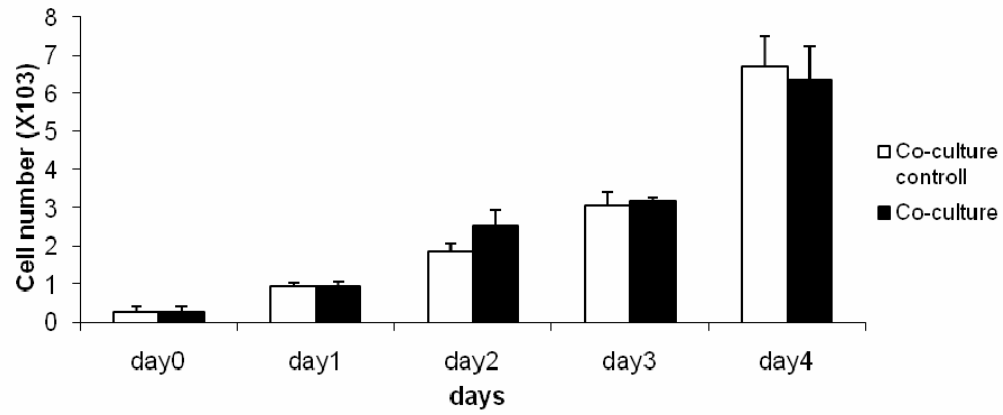
Effects of adiponectin siRNA transfection of human adipocytes

Human adipocytes were transfected with Pre-Designed siRNA-adiponectin using siPORT™ Lipid Transfection Agent as carrier. Highest transfection efficacy (70%) was obtained using the siRNA at a concentration of 80 nM in 0.6% (v/v) siPORT™ Lipid Transfection Agent without any detectable effects on cell proliferation or differentiation (data not shown).

RNA interference experiment

After transfection with adiponectin siRNA or negative control siRNA, human adipocytes were co-cultured with Caco-2 cells for 4 days. As shown in Fig. 15, Silencing adiponectin reversed the inhibitory effect of adipocytes on Caco-2 cell proliferation through day 4, and an increase in cell proliferation was observed in day 3.

A



B

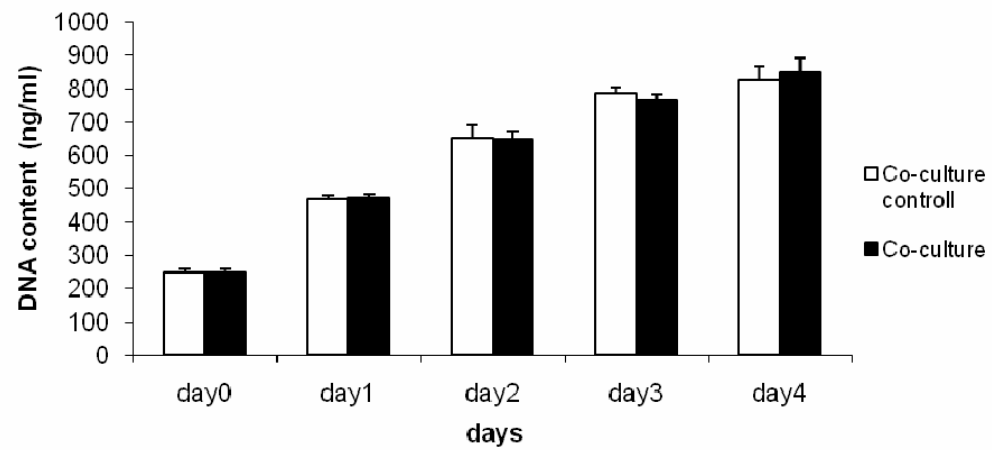


Fig. 13 Effects of co-culturing with human pre-adipocytes on Caco-2 cell proliferation

A: cell number was counted by hemocytometer

B: DNA content was determined by CyQUANT cell proliferation Kit.

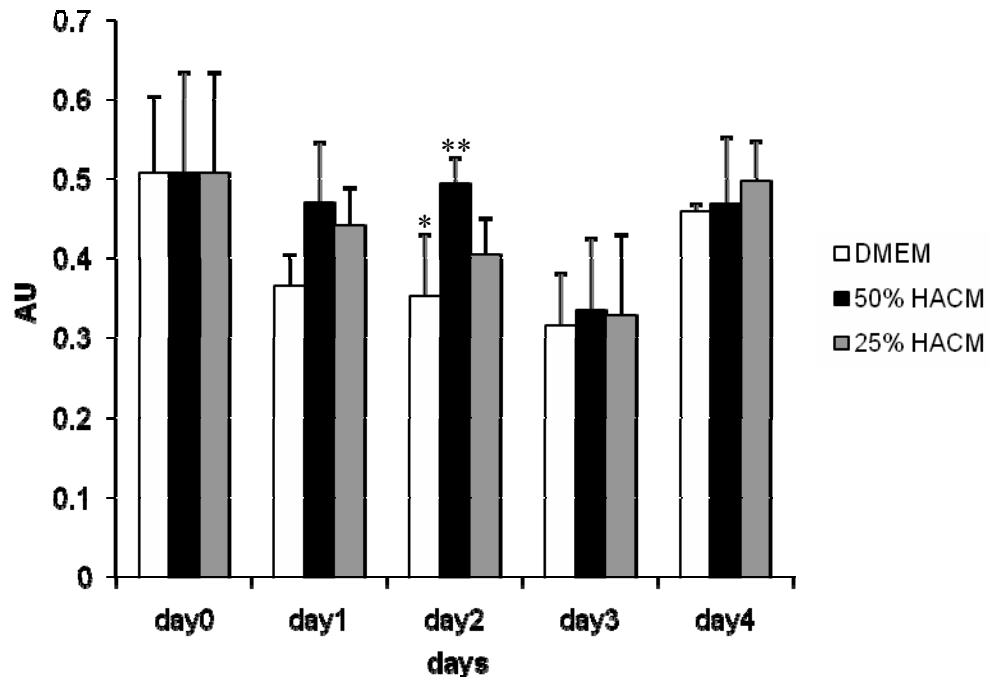
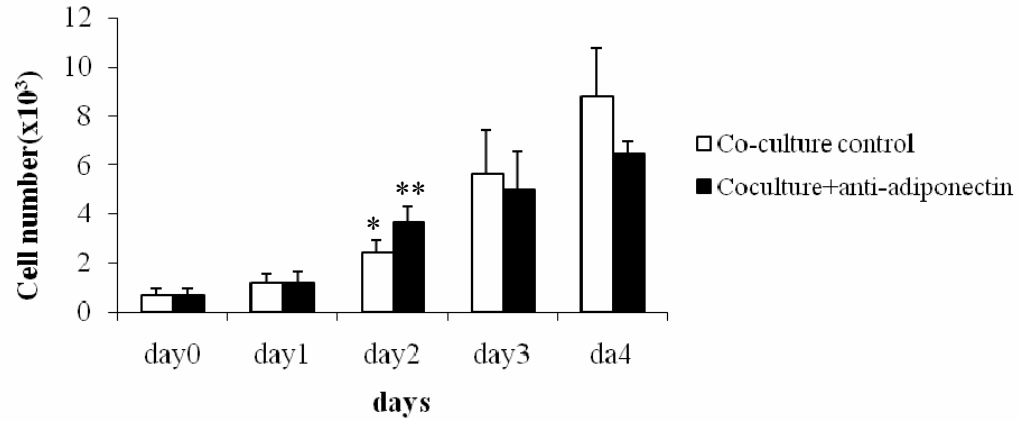


Fig. 14A Effects of anti-adiponectin treatment on HACM inhibition of Caco-2 cell proliferation

* Value is significantly different from ** value by One-way ANOVA ($p < 0.05$)

B-1



B-2

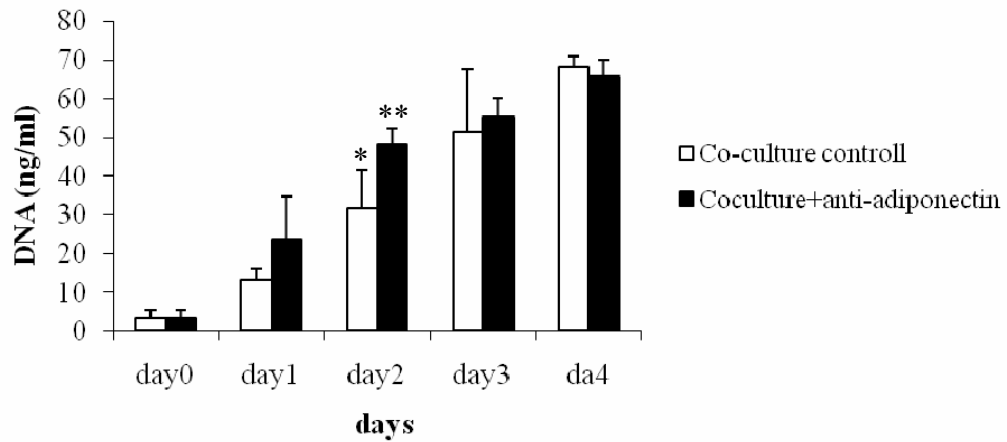


Fig.14B Effects of anti-adiponectin treatment on adipocyte co-culture inhibition of Caco-2 cell proliferation **B-1:** cell number was counted by hemocytometer

B-2: DNA content was determined by CyQuant cell proliferation Kit.

* Value is significantly different from ** value by One-way ANOVA ($p < 0.05$)

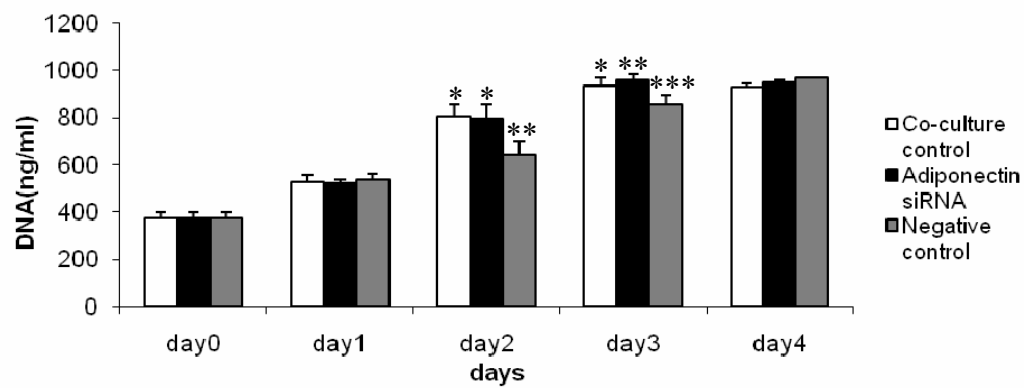


Fig. 15 Effect of adiponectin siRNA on Caco-2 cell proliferation during co-culture with human adipocytes

* Value is significantly different from ** value by One-way ANOVA ($p < 0.05$)

** Value is significantly different from *** value by One-way ANOVA ($p < 0.05$)

2.5 Discussion

Data from this study demonstrate that the anti-proliferative signal derived from human adipocytes appears to be mediated by adiponectin. Adipocytes, a predominant stromal cell type, can secrete adipokines and growth factors and thereby significantly regulate growth of epithelial cells. To date, several growth-promoting adipokines have been well characterized. For example, leptin is a small adipocyte-derived hormone, and Hardwick et al. demonstrated that leptin had mitogenic properties and can stimulate growth of colon cancer cells (15). Furthermore, a Norwegian study found a positive association between incident colorectal cancer risk and serum levels of leptin (16). TNF- α , a candidate gene for obesity and insulin resistance, is a pro-inflammatory molecule. It may be involved in the pathway linking obesity to colorectal cancer. Hepatocyte growth factor (HGF), synthesized and secreted from mature adipocytes, also shows the ability of promoting *in vitro* growth of breast cancer cells (17). In this study, we have shown for the first time that adipocyte conditioned medium, but not pre-adipocyte conditioned medium, inhibits Caco-2 cell proliferation. Adipocytes are believed to stem from fibroblast-like stem cells (preadipocytes) present in the stromal-vascular fraction (18, 19) of adipose tissue. Therefore, it is possible to observe the specific effect of adipocytes on Caco-2 by using preadipocytes as a control. Interestingly, our data show that the growth-inhibiting effect of mature adipocytes is not dose-dependent. HACM in lower concentration (25%) suppressed Caco-2 cell proliferation much stronger than the higher concentration (50%). Since both

pro-proliferative factors and anti-proliferative factors exist in HACM, these data suggest a complex interaction among these factors.

Unlike human adipocytes and HACM, human pre-adipocytes and HPCM did not show an inhibitory effect on Caco-2 cells proliferation. This difference must clearly be due to factor(s) that are derived from mature adipocytes, but not pre-adipocytes; adiponectin is well established as one such factor (20).

Accumulating epidemiological evidence links adiponectin inversely to risk of cancers of the endometrium (21-23), breast (24, 25) and colon (10, 11), and recent laboratory studies support an anti-tumor activity of adiponectin. Brakenhielm et al. reported a unique function of adiponectin as a negative regulator of angiogenesis, which is critical for tumor growth (26). Adiponectin has also been shown to drastically suppress the growth of myelomonocyte leukaemia cell lines and to induce apoptosis in these cell lines (5). Moreover, there have been two recent studies report that adiponectin is capable of suppressing cell growth in MDA-MB-231 (27, 28) and in MCF-77 (29) breast cancer cell line. However, the mechanisms that underlie adiponectin and colon carcinogenesis have not been clarified. In our pilot experiment, we found that treatment of Caco-2 cells with 5 μ g/ml human recombinant adiponectin, mimicking the human plasma adiponectin level (9), suppressed Caco-2 cell proliferation. Thus, we hypothesized that adiponectin might be responsible for the growth-inhibiting effect of HACM.

To further determine whether adiponectin contributes to this inhibitory effect, neutralizing antibody against adiponectin was used. We selected the level of antibody (150 ng/ml) based on the level of adiponectin in HACM, and the data

showed that anti-adiponectin almost completely abrogated the growth-inhibitory activity of HACM.

The co-culture system is a model that focuses on stromal-cancer interactions. Although *in vivo* studies illustrate that characterize human epithelial cells require the presence of human adipose tissue, most *in vitro* studies used either 3T3-L1 CM (17, 30) or 3T3-L1 adipocytes (31), or mammary mature adipocytes (32). To our knowledge, our experiments are the first to utilize viable mature human adipocytes in co-culture with colon cancer cells. In the current study, we used a transwell insert system to physically separate the human adipocytes and Caco-2 cells, while still allowing the free exchange of low molecular weight factors. In agreement with previous experiments, we found that adipocytes inhibited Caco-2 cell proliferation and that neutralizing adiponectin with an antibody reversed this effect.

RNA interference is now an established technology that is widely used for highly specific gene silencing in various cell types (33). Previous studies identified conditions for siRNA-based gene silencing in cultured 3T3-L1 adipocytes (34-36), but had not yet been generally applied to human adipocytes. In the present study, we first optimized the siRNA conditions for human adipocytes to knock down adiponectin gene expression for 5 days. We achieved a 70% knock down of adiponectin and the efficiency was maintained throughout the experiment, as demonstrated by measuring adiponectin levels from day 1 to day 4. When introduced in the co-culture system, silencing adiponectin not only reversed the growth-inhibiting effect of human adipocytes on Caco-2

proliferation, but also increased Caco-2 cell growth, similar to the anti-adiponectin results. These results indicate that adiponectin contributes to the regulation of Caco-2 cell proliferation, and adiponectin knockdown-human adipocytes stimulated Caco-2 proliferation.

In summary, we have demonstrated human adipocytes, but not pre-adipocytes, inhibit Caco-2 cell proliferation. This inhibitory effect appears to be due to adiponectin secreted by mature adipocytes. Our results have led to a new insight into the role of adiposity in colorectal tumorigenesis. Further work will be necessary to fully characterize the biology of this inhibitory effect.

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PART 4
CONCLUSIONS

CONCLUSIONS

The strong association between high calcium intake and lower colorectal cancer risk, and between obesity and higher colorectal cancer risk have been generally accepted. However, studies presented here support the hypothesis that increased intestinal tumorigenesis in $Apc^{Min/+}$ mice on high calcium diets is due to the extreme loss of body fat, therefore providing a new perspective on the role of adipose tissue; that is, a minimum amount or threshold level of adipose tissue may be required to significantly attenuate the augmented tumorigenic response observed in the $Apc^{Min/+}$ mice on a high calcium diet. Our studies also suggested that tumor load was not significantly different in $A^y/Apc^{Min/+}$ mice fed the low, normal and high calcium diets despite the significant inverse relationship between dietary calcium and level of adipose tissue was observed, further suggesting that adequate level of body fat may protect against excessive intestinal tumor load.

Adipokines are likely candidates to explain the role of adipose tissue in the incidence of colorectal cancer. Data from present study demonstrate that human adipocytes have the ability to inhibit human Caco-2 cell proliferation via an adiponectin-dependent mechanism. Accordingly, loss of this effect in lipodystrophic *min* mice fed high calcium diet may be responsible for observed increases in tumor load.

In conclusion, we demonstrate that the increased intestinal tumor susceptibility is partially due to the loss of adiponectin from excessive loss of body fat in *min* mice on high calcium diets. Further studies of the precise role of adiponectin in regulating tumorigenesis should be conducted in both *in vivo* and *in vitro*

experimental models. We hypothesize that the protective effect of adiponectin could be achieved by reducing cell proliferation, improving insulin resistance and inflammatory status.

VITA

Shengli Ding was born in January 1977 in NanJing, China. After graduated from Jing-Ling high school, she attended NanJing Medical University in 1995 and earned her M. D. degree in 2000. In the same year, she continued her study in NanJing Medical University and earned Master of Science degree in Pediatrics in 2003. Shengli began her doctoral studies in the fall of 2003 at the University of Tennessee, Knoxville. She defended her dissertation in May 2007.