Adiposity-Related Protection of Intestinal Tumorigenesis: Interaction With Dietary Calcium

S. Ding, M. F. McEntee, J. Whelan, and M. Zemel

Abstract: Although high-calcium diets have been reported to reduce the risk of colorectal cancer, our preliminary data with the adenomatous polyposis coli (Apc) Min mutation (Min/+; ApcMin/+ ) 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 ApcMin/+ 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 ApcMin/+ mice were crossed with obesity prone lethal yellow agouti (A+/a) mice to generate obese A+/ApcMin/+ mice. Low (0.2%), normal (0.5%), and high (1.2%) calcium diets were fed to both A+/ApcMin/+ mice and ApcMin/+ 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+/ApcMin/+ (P < 0.004) and by 65–82% in ApcMin/+ (P < 0.03). ApcMin/+ 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+/ApcMin/+ mouse. β-Catenin and cyclin D1 gene expression were significantly induced with high calcium diet in intestinal tumor tissue of ApcMin/+ mice but not in A+/ApcMin/+ mice. We conclude that the differential effect of dietary calcium on intestinal tumorigenesis in lean vs. obese ApcMin/+ may result from the loss of adipose-derived protective factor(s) due to the substantial loss of body fat in ApcMin/+ mice fed a high calcium dairy diet, increasing β-catenin and cyclin D1 in tumors.

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 (1) published data indicating that dietary calcium was inversely associated with epithelial proliferation. 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 adenomatous polyposis coli (Apc) Min mutation (Min/+; C57BL/6J-ApcMin/+ ) mouse is highly susceptible to spontaneous intestinal adenoma formation due to a heterozygous, dominant mutation in the Apc 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 initiate tumorigenesis in familial adenomatous polyposis and sporadic colorectal cancer (9,10). The ApcMin/+ 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 ApcMin/+ 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 Go (13) did not find an effect of a high calcium diet on polyp number or tumor load over the entire intestine in female ApcMin/+ mice. In contrast, our preliminary observations suggested that dietary calcium intake paradoxically promoted intestinal tumor growth in ApcMin/+ 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 an increase in tumor load.
in increased tumor susceptibility in \textit{Apc}^{Min/+} mice on the high calcium dairy diet.

Agouti mutations result in ectopic expression of the agouti protein in mice. Lethal yellow (\textit{Ay}) mutation is one of the dominant agouti mutations. It is characterized by embryonic lethality of the homozygous \textit{A}^{\text{y}}/\textit{A}^{\text{y}} genotype (16). The \textit{A}^{\text{y}} genotype results from a large deletion that includes the coding regions of the heterogeneous nuclear ribonucleo-protein associated with lethal yellow (\textit{raly}) gene. Consequently, the \textit{A}^{\text{y}} allele is under the control of the \textit{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 \textit{Apc}^{Min/+} mice fed the high calcium dairy diet was associated with elevated tumor load, while this was not the case in \textit{A}^{\text{y}}/\textit{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.

\section*{Materials and Methods}

\subsection*{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-\textit{Apc}^{Min/+} (Jackson Laboratory, Bar Harbor, ME) at 6–7 wk of age and female C57BL/6J-\textit{A}^{\text{y}} mice to generate \textit{A}^{\text{y}}/\textit{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 Hot sodium Hydroxide and Tris extraction protocol (18) and a protocol associated with lethal yellow (\textit{Ay}) gene. Consequently, the \textit{Ay} genotype results from a large deletion that includes the coding region of the \textit{Min} gene. The \textit{Min} allele is under the control of the \textit{raly} promoter, and ec-topic overexpression of the agouti protein produces the effects associated with lethal yellow coat color, obesity, diabetes, and tumor susceptibility (16,17).

\section*{Experimental Design}

\textit{A}^{\text{y}}/\textit{Apc}^{Min/+} and \textit{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 3 levels of calcium (0.2%, 0.5%, or 1.2% wt/wt) either in the form of carbonate calcium (0.2% and 0.5%) or nonfat 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 (Research Diets, Inc., NJ) 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 pentobarbital (50 mg/kg body weight). Plasma samples were collected via cardiac puncture and kept at \(-20^\circ\text{C}\). To determine the number and size of intestinal tumors, the entire intestinal tract was removed, flushed with ice cold phosphate-buffered saline and opened longitudinally as described previously (20). Tumor number and size were determined using a dissecting microscope (x18) (20). Four fat depots (abdominal, subcutaneous, epididymal, and perirenal) and skeletal muscle (soleus and gastrocnemius) mass were weighted and immediately frozen at \(-80^\circ\text{C}\) with pooled samples of small intestinal tumors from individual mice.

\section*{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).

\section*{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 manufacturer’s instruction. Mouse \textit{Bax} gene, \textit{Bcl}-2 gene, \textit{β}-\textit{catenin} gene, and \textit{cyclin D1} gene expression levels of tumor intestine tissues were measured quantitatively using 7300 Real-Time polymerase chain reaction (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).

\section*{Statistical Analysis}

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

\section*{Results}

\subsection*{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 \textit{A}^{\text{y}}/\textit{Apc}^{Min/+} and \textit{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 \textit{A}^{\text{y}}/\textit{Apc}^{Min/+} and \textit{Apc}^{Min/+}, respectively.
Table 1. Average Food Consumption (g/day) of Ay/ApcMin/+ and ApcMin/+ Micea

<table>
<thead>
<tr>
<th>Diet</th>
<th>Ay/ApcMin/+ Mice</th>
<th>ApcMin/+ Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low calcium</td>
<td>4.00 ± 0.11</td>
<td>3.47 ± 0.10</td>
</tr>
<tr>
<td>Medium calcium</td>
<td>3.90 ± 0.10</td>
<td>3.03 ± 0.12</td>
</tr>
<tr>
<td>High calcium dairy</td>
<td>4.16 ± 0.17</td>
<td>3.28 ± 0.24</td>
</tr>
</tbody>
</table>

aData expressed as mean ± SE.

Table 2. Body Weight Gain (g) (in 5–6 wk) of Ay/ApcMin/+ and ApcMin/+ Mice in Different Diet Groupsa

<table>
<thead>
<tr>
<th>Diet</th>
<th>Ay/ApcMin/+ Mice</th>
<th>ApcMin/+ Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low calcium diet</td>
<td>13.38 ± 1.51</td>
<td>2.64 ± 0.85</td>
</tr>
<tr>
<td>Medium calcium diet</td>
<td>8.78 ± 1.72</td>
<td>1.95 ± 0.74</td>
</tr>
<tr>
<td>High calcium dairy</td>
<td>4.89 ± 1.76*</td>
<td>0.03 ± 0.95</td>
</tr>
</tbody>
</table>

aData expressed as mean ± SE. *P < 0.01 vs. low calcium diet group in Ay/ApcMin/+ 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 Ay/ApcMin/++; P = 0.023 in ApcMin/+). Feeding the Ay/ApcMin/+ 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, Table 3) compared to the low calcium diet. This decrease was also found in the ApcMin/+ mice in which the high calcium dairy diet caused decreases in 3 fat masses 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 Ay/ApcMin/+ mice (Table 4). A lack of effect was also observed in ApcMin/+ mice fed the low and medium levels of calcium. However, when ApcMin/+ 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.

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 Ay/ApcMin/+ mice, P < 0.001; r = −0.450 in ApcMin/+ mice, P < 0.002) (Fig. 1).

Effects of Calcium Diets on Plasma Leptin and Insulin Levels

Plasma leptin levels in the Ay/ApcMin/+ 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 vs. 51.4 ± 7.6 ng/ml, respectively), but diet had no effect on serum leptin levels in the ApcMin/+ mice (Fig. 2). Two-way ANOVA analysis showed that the overall plasma leptin levels were higher in the Ay/ApcMin/+ mice as compared to ApcMin/+ mice with all levels of dietary calcium (P < 0.01).

Figure 1. Correlation between body fat mass and tumors. A: Ay/ApcMin/+ mice. B: ApcMin/+ mice.

Figure 2. Effects of calcium diets on plasma leptin levels of Ay/ApcMin/+ (+) mice and ApcMin/+ mice (+). *P < 0.02 vs. low calcium diet group in Ay/ApcMin/+ mice, P < 0.001 vs. Ay/ApcMin/+ mice within low calcium diet group.
Table 3. Effects of Different Calcium Diets on Fat Mass and Skeletal Musclesa,b

<table>
<thead>
<tr>
<th></th>
<th>Aβ/Apc&lt;sup&gt;Min+/−&lt;/sup&gt; Mice</th>
<th></th>
<th>Apc&lt;sup&gt;Min+/+&lt;/sup&gt; Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2% Calcium (n = 7)</td>
<td>0.5% Calcium (n = 7)</td>
<td>1.2% Calcium (n = 7)</td>
</tr>
<tr>
<td>Abdominal</td>
<td>1.92 ± 0.19</td>
<td>1.47 ± 0.23</td>
<td>1.03 ± 0.28</td>
</tr>
<tr>
<td>Perirenal</td>
<td>0.92 ± 0.11</td>
<td>0.63 ± 0.09</td>
<td>0.39 ± 0.07</td>
</tr>
<tr>
<td>Epididymal</td>
<td>0.16 ± 0.02</td>
<td>0.16 ± 0.04</td>
<td>0.14 ± 0.04</td>
</tr>
<tr>
<td>Subcutaneous</td>
<td>2.93 ± 0.24</td>
<td>1.96 ± 0.26</td>
<td>1.38 ± 0.32</td>
</tr>
<tr>
<td>Total fat mass</td>
<td>5.86 ± 0.51</td>
<td>4.18 ± 0.58</td>
<td>2.87 ± 0.61</td>
</tr>
<tr>
<td>Soleus</td>
<td>0.01 ± 0.004</td>
<td>0.01 ± 0.005</td>
<td>0.01 ± 0.005</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>0.05 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.06 ± 0.01</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data expressed as mean ± SE.
<sup>b</sup> Values in each row with different symbols (*, †) are significantly different by 1-way analysis of variance (ANOVA), P < 0.05.
<sup>c</sup> Value is significantly different from § value by 1-way ANOVA, P < 0.05.

Dietary calcium exerted no effect on plasma insulin levels in either Aβ/Apc<sup>Min+/−</sup> or Apc<sup>Min+/+</sup> mice (Fig. 3); however, the overall insulin levels of Apc<sup>Min+/+</sup> mice were higher than that of Aβ/Apc<sup>Min+/−</sup> mice at each level of calcium diet (P < 0.001).

Effects of Calcium Diets on Intestinal Tumor β-Catenin and Cyclin D1 Gene Expression

In Aβ/Apc<sup>Min+/+</sup> mice intestinal tumor, there were no significant differences in β-catenin levels between the high and low calcium diets (Fig. 4).

In contrast, Apc<sup>Min+/+</sup> mice on the high calcium diet had tumors with 142% higher levels of β-catenin gene expression (Fig. 5D; P < 0.05) in comparison to 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. 6.)

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, are 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 calcium-sensing receptors (CaSR) 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)-D<sub>3</sub>], effects as a first messenger (i.e., via CaSR), 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 that 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 yr) calcium supplementation on the incidence of colorectal cancer among postmenopausal 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, a dietary formula for gestating and growing rodents as recommended by the American Society for Nutrition (27), was modified with 3 levels of dietary calcium. The typical AIN-93G diet contains 0.5% calcium by weight or 1.2 mg/kcal. If one assumes an equivalency

![Figure 3. Effects of calcium diets on plasma insulin levels of Aβ/Apc<sup>Min+/−</sup> (+) mice and Apc<sup>Min+/+</sup> mice(+).](image-url)
Figure 4. 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: \( \beta\)-catenin.

Figure 5. Effects of calcium diets on \( Apc^{Min/}\) mice intestinal tumor tissue gene expression. A: Bax. B: Bcl-2. C: Bcl-2/Bax ratio. D: \( \beta\)-catenin. Means without a common letter (a, b) differ by 1-way analysis of variance, \( P < 0.05\).
Table 4. Effects of Different Calcium Diets on Tumor in the Colon and Intestine of Ay/ApcMin/+ Mice and ApcMin/+ Mice

<table>
<thead>
<tr>
<th></th>
<th>Ay/ApcMin/+ Mice</th>
<th></th>
<th></th>
<th>ApcMin/+ Mice</th>
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<tbody>
<tr>
<td></td>
<td>0.2% Calcium</td>
<td>0.5% Calcium</td>
<td>1.2% Calcium</td>
<td>0.2% Calcium</td>
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<tr>
<td></td>
<td>(n = 7)</td>
<td>(n = 7)</td>
<td>(n = 7)</td>
<td>(n = 7)</td>
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<tr>
<td>Colon</td>
<td></td>
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<tr>
<td>&lt;1 mm</td>
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<td></td>
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<td>店名称</td>
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<td>1.01–2 mm</td>
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<td>店名称</td>
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<tr>
<td>2.01–3 mm</td>
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<td>店名称</td>
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<tr>
<td>3.01–4 mm</td>
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<td>店名称</td>
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<tr>
<td>&gt;4 mm</td>
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<td>店名称</td>
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<tr>
<td>Small intestine</td>
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<tr>
<td>&lt;0.5 mm</td>
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<td>0.51–1 mm</td>
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<tr>
<td>1.01–1.50 mm</td>
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<tr>
<td>1.51–2 mm</td>
<td></td>
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<td>店名称</td>
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<tr>
<td>2.01–2.5 mm</td>
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<tr>
<td>2.51–3 mm</td>
<td></td>
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<td>店名称</td>
</tr>
<tr>
<td>&gt;3 mm</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Total tumor number/group</td>
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<td>301</td>
<td>300</td>
<td>店名称</td>
</tr>
<tr>
<td>Average tumor/mouse</td>
<td>33.43 ± 2.65</td>
<td>42.86 ± 8.66</td>
<td>47.71 ± 2.55</td>
<td>店名称</td>
</tr>
<tr>
<td>Average tumor size</td>
<td>1.21 ± 0.45</td>
<td>1.31 ± 0.48</td>
<td>1.33 ± 0.35</td>
<td>店名称</td>
</tr>
</tbody>
</table>

*a* None observed.

*b* Number of tumors.

*c* Data expressed as mean ± SE.

*d* Values in row with different symbols (*, †) are significantly different by 1-way analysis of variance, \( P < 0.05. \)

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 (United States) (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 (United States). 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

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![Figure 6](image_url)  
**Figure 6.** Effects of calcium diets on ApcMin/+ mice intestinal tumor cyclin D1 gene expression in ApcMin/+ mice. *P < 0.05 vs. low and medium calcium diet group.
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 threefold higher than the lowest level used in this study).

This study demonstrated that a high calcium diet surprisingly resulted in a pro-tumorigenic effect that may not be related to previously reported mechanisms of calcium but rather indirectly associated with its antiobesity effects. The amount of calcium and the vehicle of the supplementation appear to be critical for this response, particularly in \textit{Apc} \textit{Min}/+ mice, an accepted model of intestinal tumorigenesis that has been shown to be sensitive to dietary intervention (31–34). Previously, it had been demonstrated that providing \textit{Apc} \textit{Min}/+ mice variable levels of elemental calcium in the form of calcium carbonate (0.2–1.2\%, wt/wt) 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 nonfat dry milk, tumor number was 52\% higher in animals provided a high dose of calcium (1.2\%, wt/wt) vs. a low dose of calcium (0.2\%, wt/wt calcium carbonate; an average of 62 tumors/mouse vs. 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) yet have larger fat pad mass compared to \textit{Apc} \textit{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 \textit{Apc} \textit{Min}/+ mice versus the \textit{Apc} \textit{Min}/+ mice. Recapitulating our earlier results, tumor number was significantly higher in the \textit{Apc} \textit{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 \textit{Apc} \textit{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 \textit{Apc} \textit{Min}/+ mice on the high calcium diets. If this were the case, the differential effect of dietary 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 1 or more of these adipokines (i.e., leptin, resistin, adiponectin, TNF-\(\alpha\), 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 A1/ApcMin/+ mice were significantly heavier with larger fat pad mass than their ApcMin/+ counterparts, and this difference was reflected in higher circulating levels of leptin, a biomarker of adipose tissue (Fig. 2). We also observed that insulin levels in ApcMin/+ mice were higher than that of A1/ApcMin/+ 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 ApcMin/+ on the high dairy calcium diet.

B-catenin expression was enhanced in ApcMin/+ 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 ApcMin/+ mice on a high calcium dairy diet (Fig. 6) 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 ApcMin/+ 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 ApcMin/+ mouse with obesity-prone A1/a mouse to generate a “fat” A1/ApcMin/+ 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).

Acknowledgments and Notes

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