

Research Article

Dietary Methyl Donor Depletion Protects Against Intestinal Tumorigenesis in *Apc^{Min/+}* MiceKrishna Kadaveru¹, Petr Protiva^{1,2}, Emily J. Greenspan¹, Young-In Kim³, and Daniel W. Rosenberg¹

Abstract

Despite recent population data, the influence of dietary folate supplementation on colon cancer risk remains controversial. This study examines the effects of folate deficiency, in combination with choline, methionine, and vitamin B12 depletion, on intestinal tumorigenesis in *Apc^{Min/+}* mice. Methyl donor sufficient (MDS) and deficient (MDD) diets were started at five or 10 weeks of age and tumors evaluated at 16 weeks. MDD suppressed intestinal tumor formation in *Apc^{Min/+}* mice (~80%) when started at five weeks of age. The protective effect was lost when MDD was initiated at 10 weeks of age, indicating an important time dependency on cancer suppression. Concomitant with cancer protection, MDD restricted body weight gain. Therefore, a second study was conducted in which MDS was given *ad libitum* or pair-fed with MDD. Although small intestinal tumors were reduced 54% in pair-fed MDS mice, MDD caused a further reduction (96%). In colon, although MDD did not affect tumor numbers, tumor size was reduced. Gene expression profiling of normal-appearing colonic mucosa after 11 weeks on MDD identified a total of 493 significantly down-regulated genes relative to the MDS group. Pathway analysis placed many of these genes within general categories of inflammatory signaling and cell-cycle regulation, consistent with recently published human data obtained during folate depletion. Further studies are warranted to investigate the complex interplay of methyl donor status and cancer protection in high-risk populations. *Cancer Prev Res*; 5(7); 911–20. ©2012 AACR.

Introduction

Epidemiologic studies have suggested that low folate levels are associated with an increased risk of colorectal cancer (1–5) and other health risks such as congenital neural tube defects. To reduce the incidence of neural tube defects, the U.S. Food and Drug Administration instituted a mandatory folate fortification program in 1998, leading to near doubling of median folate levels. Because folate is an essential cofactor in DNA methylation and nucleotide synthesis, there is ongoing concern that folate fortification may lead to increased risk for some forms of cancer, particularly among subjects harboring neoplastic foci. Indeed, early research in the 1940s showed that administration of folic acid to patients with malignancy actually results in rapid progression of the disease (6). One recent clinical study showed

acceleration in the development of premalignant adenomas (7), and 2 other clinical trials observed an increase in cancer incidence (8, 9) among subjects randomly assigned to receive folic acid supplements. Nevertheless, 2 contemporary postfortification studies showed that compared with individuals on relatively low folate diet, individuals on adequate folate diets had reduced risk of colorectal cancer (10, 11), suggesting that adverse effects of folate might be limited to populations consuming large amounts of folic acid.

The folate controversy stems in part from the relative lack of mechanistic data about specific cellular pathways through which either low or high folate intake modulates the risk of colorectal cancer. We recently showed in human subjects that changing folate availability by dietary depletion or supplementation alters the expression of a number of genes involved in the inflammatory and immune response in the colonic mucosa (12). Another human study showed that the administration of 1.2 mg folic acid for 12 weeks increases the levels of serum proteins involved in the regulation and activation of immune function and the complement cascade (13). In addition, in the Aspirin/Folate Prevention of Large Bowel Polyps trial, folic acid administration was shown to antagonize the suppressive effects of aspirin on circulating inflammatory markers (14). These folate-associated molecular changes may provide clues as to how folate status might modulate the risk of colorectal carcinogenesis and neoplastic progression, but additional studies examining tumor outcomes are needed.

The aim of this study was to determine the effects of folate/methyl donor depletion on intestinal tumorigenesis

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in $Apc^{Min/+}$ mice. We hypothesized that folate/methyl donor depletion would attenuate the formation of intestinal tumors and, based on our recently published human data (12), reduce the expression of a panel of immune-related genes within the colonic mucosa. The use of antibiotics was avoided, thus preventing the severe methyl donor deficiency associated with suppression of the gut microflora (15, 16). Overall, our data show that folate/methyl donor deficiency markedly suppresses intestinal tumorigenesis in $Apc^{Min/+}$ mice. The effect is not solely dependent on reduced caloric intake, however, as further shown by a pair-feeding study with MDS diet. Importantly, the protection afforded to the intestine is highly dependent upon the timing and duration of the dietary intervention.

Materials and Methods

Animals, diets, and experimental design

Animal experiments were conducted with approval from the Center for Laboratory Animal Care Committee (CLACC), The University of Connecticut Health Center. $Apc^{Min/+}$ mice, originally purchased from The Jackson Laboratory, were maintained on a C57BL/6J background. Genotyping was carried out by tail biopsy using PCR-based assays to determine the *Apc* gene status. As outlined in Fig. 1A, in study 1, a total of 39 $Apc^{Min/+}$ mice were randomized at 5 weeks of age and placed into 4 experimental groups. In groups 1 and 2, experimental diets were initiated at 5 weeks of age. In group 1, 12 $Apc^{Min/+}$ mice were fed a methyl donor sufficient (MDS) diet (TD.99366; Harlan Laboratories) for a total of 11 weeks. In group 2, 14 $Apc^{Min/+}$ mice were fed a methyl donor deficient (MDD) diet (TD.00605), devoid of folate, choline, methionine, and vitamin B12 (complete details of the diets are available in Supplementary Table S1) for a total of 11 weeks. In group 3, a total of 5 $Apc^{Min/+}$ mice received the MDS beginning at 10 weeks of age, whereas in group 4, 8 $Apc^{Min/+}$ mice received the MDD beginning at 10 weeks of age. In study 2, as outlined in Fig. 1B, a total of 37 $Apc^{Min/+}$ mice were randomized at 5 weeks of age and placed into 3 experimental groups. In each group, experimental diets were initiated in mice at 5 weeks of age for a total of 11 weeks. In group 1, $Apc^{Min/+}$ mice ($n = 13$) were fed the MDS *ad libitum*. In group 2, $Apc^{Min/+}$ mice ($n = 12$) were fed a MDD diet *ad libitum*. Finally, in group 3, to prevent weight differences between the experimental and control diets, $Apc^{Min/+}$ mice ($n = 12$) were pair-fed with MDS diet to the same amount consumed by the group 2 animals. All mice in each group were sacrificed at 16 weeks of age. Body weights of mice were recorded weekly and reported as shown in Fig. 2, Supplementary Fig. S1, and Fig. 3.

Serum folate levels

At the time of sacrifice, blood was collected from the posterior vena cava and centrifuged at $10,000 \times g$ for 10 minutes at 4°C . Serum was collected and stored at -80°C until analysis. Serum folate concentrations were measured by a microtiter plate assay using *Lactobacillus casei*, as described previously (17).

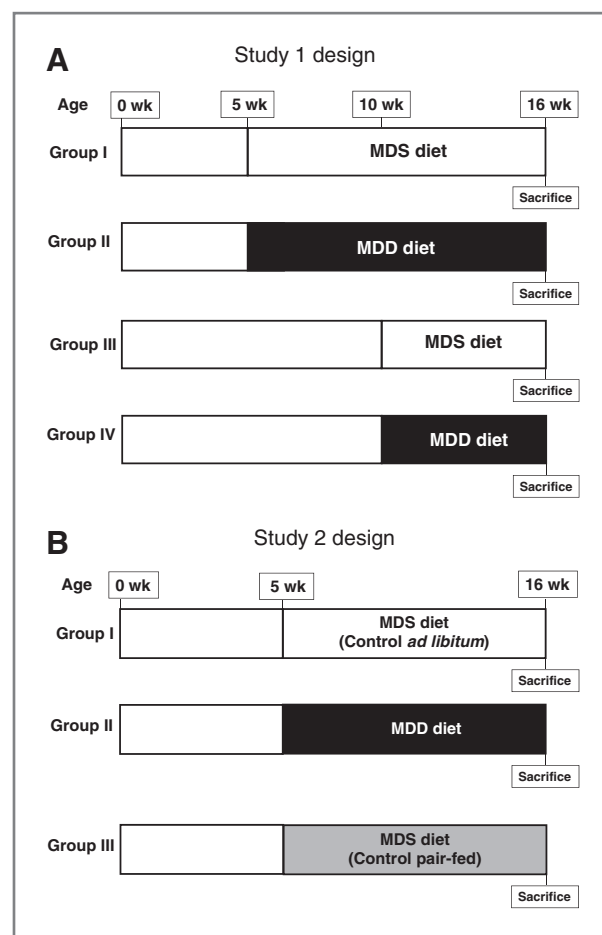


Figure 1. Study design. A, study 1, at 5 weeks of age, a total of 39 $Apc^{Min/+}$ mice were randomized and placed into 4 experimental groups. Group I ($n = 12$) received MDS diet and group II ($n = 14$) received MDD diet for 11 weeks starting at 5 weeks of age. Group III ($n = 5$) received MDS diet and group IV ($n = 8$) received MDD diet for 6 weeks starting at 10 weeks of age. All mice were sacrificed at 16 weeks of age. B, study 2, at 5 weeks of age, a total of 37 $Apc^{Min/+}$ mice were randomized and placed into 3 experimental groups. Group I (MDS *ad libitum*, $n = 13$) received MDS diet *ad libitum*, group II (MDD, $n = 12$) received MDD diet and group III (MDS pair-fed, $n = 12$) were pair-fed with MDS diet to the same amount consumed by the group II animals for 11 weeks starting at 5 weeks of age. All mice were sacrificed at 16 weeks of age.

Tumor incidence and multiplicity

Mice were sacrificed at 16 weeks of age. The small intestine and colon were harvested, flushed immediately with ice-cold PBS, and slit open longitudinally. Specimens were fixed flat in 10% buffered formalin and stored in 70% ethanol. For tumor quantitation, fixed tissues were stained with 0.2% methylene blue and examined under a dissecting microscope.

RNA extraction and gene expression analyses

Sections of excised colons with no macroscopic evidence of tumors from the study 1 MDS group 1 ($n = 3$) and MDD group 2 ($n = 5$) were maintained in liquid nitrogen until analysis. Total RNA was extracted using the Trizol Reagent

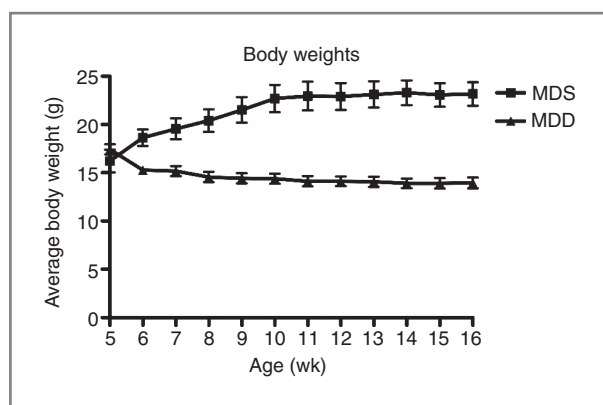


Figure 2. Body weight changes in *Apc^{Min/+}* mice maintained for 11 weeks on MDS or MDD diet (study 1). Mice were placed on MDS or MDD diet beginning at 5 weeks of age. Body weights were recorded weekly throughout the entire experimental period. Each data point represents the average body weight \pm SEM for group I ($n = 12$) and group II ($n = 14$). Note that at the end of the study, there was an approximately 40% reduction in body weight in mice maintained on the MDD diet.

(Invitrogen). All RNA samples were assessed for quality on an Agilent 2100 Bioanalyzer (Agilent Technologies). The Microarray Core at Children's Hospital (Boston, MA) carried out array processing and cRNA hybridization. Briefly, total RNA was converted into cRNA labeled with biotin and hybridized onto the Mouse-Ref8 v1.1 Sentrix BeadChip Array (Illumina Corp.) for approximately 18 hours. The BeadChip Array was then washed and stained with Streptavidin-Cy3 and scanned by the Illumina BeadArray Reader (Illumina Corp.). Each sample was hybridized to 2 separate BeadChips. Images were quantified using the Illumina BeadStudio software and values were reported as average signal for each gene.

Expression data were analyzed by Genespring software (Agilent Technologies, Inc.) after normalization. Before

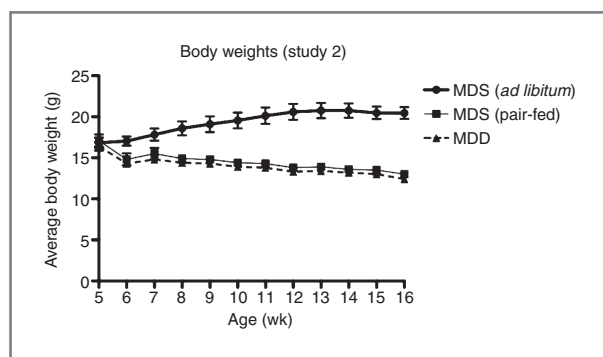


Figure 3. Body weight changes in *Apc^{Min/+}* mice maintained for 11 weeks on MDS or MDD diet (study 2). Mice were placed on MDS or MDD diet beginning at 5 weeks of age. Body weights were recorded weekly during the entire experimental period. Each data point represents the average body weight \pm SEM for group I (MDS *ad libitum*, $n = 13$), group II (MDD, $n = 12$) and group III (MDS pair-fed, $n = 12$). Average body weights for group II and group III overlapped throughout the entire experimental period. Note that at the end of the study, there was an approximately 40% reduction in body weight in mice maintained on MDS pair-fed and MDD diets.

analysis, the arrays were normalized to the 50th percentile per chip (array) and median per gene. Quality control was carried out by analyzing gene expression correlation coefficients, and samples with coefficient less than 0.97 were excluded (one control and one intervention animal). Biologic duplicate samples signals were averaged. The differences in gene expression levels were determined using *t* tests, adjusted by the Benjamini-Hochberg method at a false discovery rate (FDR) of less than 0.1. The significant gene list was subjected to hierarchical clustering using squared Euclidian distance and a centroid linkage rule (18). This microarray data set has been deposited in the NCBI Gene Expression Omnibus with the accession number GSE37010. Subsequently, Gene Set Enrichment Analysis (GSEA) was used. GSEA is a computational method that determines whether an *a priori* defined set of genes show statistically significant differences between 2 phenotypes. We ranked gene expression differences between MDS and MDD groups to identify gene sets that were significantly enriched after methyl donor depletion (19, 20). FDR *Q* was used to rank the enrichment results.

Quantitative real-time analysis

For reverse transcriptase PCR (RT-PCR), duplicate 1- μ g samples of total RNA were used as a template for cDNA synthesis using Superscript III First Strand kit (Invitrogen). cDNA was diluted in RT buffer and amount corresponding to 100 ng of original RNA was used for gene expression by quantitative RT-PCR (qRT-PCR). All qRT-PCR reactions used TaqMan Gene Expression Assay probes and primers (Applied Biosystems) and the ABI Prism 7500 RT-PCR system at the Liver Center (Yale University, New Haven, CT). Three genes that are related to inflammation were chosen from among the top 10 most downregulated genes (TNF- α , CXCL1, and CD28) and were measured by qRT-PCR to validate the expression array data. Quantification of expression changes used the delta method, and 18S mRNA was used as endogenous controls. Two independent RT-PCR reactions were used for each sample to calculate results.

Statistics

Statistical analysis of the gene expression arrays is described above. For the comparison of serum folate levels, the number of intestinal polyps, and qRT-PCR data, statistical analyses were done using an unpaired *t* test, and *P* values are indicated in the figures. In study 2, one-way ANOVA with Bonferroni posttest was used to compare the number of intestinal polyps between groups. The difference in colon tumor size in response to methyl donor depletion was tested using χ^2 test (size cutoff of 2 mm).

Results

Effects of dietary methyl donor depletion on intestinal tumor formation

In study 1, *Apc^{Min/+}* mice were maintained on either the MDS or MDD diet, beginning at either 5 or 10 weeks of age (Fig. 1A). Regardless of timing, mice lost weight on the MDD diet, although the weight loss leveled off after 8 weeks

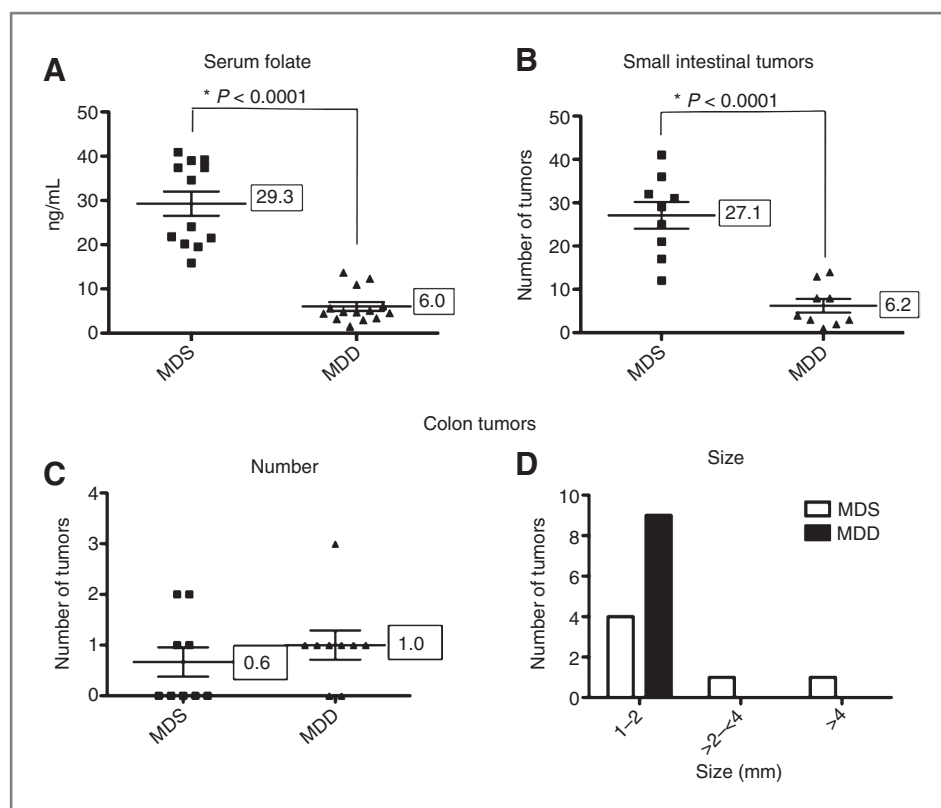


Figure 4. The effects of methyl donor depletion for 11 weeks on serum folate concentrations and intestinal tumor formation. A, serum folate concentrations were determined by a microtiter plate assay using *Lactobacillus casei*, as described under Materials and Methods; B and C, total number of tumors per mouse in the small intestine (B) and colon (C) from MDS and MDD ($n = 9$ per each group) mice, respectively. D, colon tumors were classified by size as indicated. Note that there were no medium (between 2 and 4 mm) or large-sized (≥ 4 mm) colon tumors in the MDD group. Each data point represents an individual mouse and the numbers indicate the mean value for each group. P values are indicated in the Figure and determined by unpaired t tests.

on the experimental diet (Fig. 2). At the end of the study period, there was almost a 40% body weight difference between the MDS and MDD groups (Fig. 2), consistent with previous folate depletion studies in rodents (15, 21–23). Although the MDD diet caused a significant reduction in body weight gain, there were no premature deaths recorded. To confirm the effects of dietary folate depletion, the serum concentrations were also measured. As shown in Fig. 4A, the mean serum folate concentration in the MDD group was reduced by approximately 80% compared with the MDS dietary group ($P < 0.0001$) when dietary intervention with the MDD diet was initiated at 5 weeks of age.

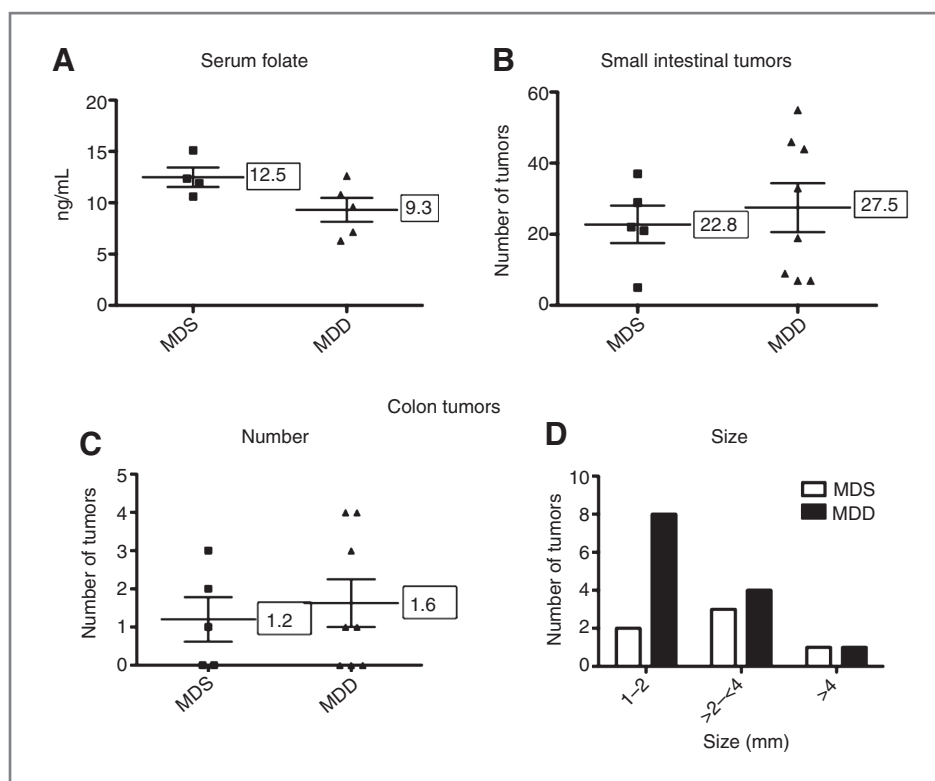
To determine the effects of methyl donor depletion on *Apc^{Min/+}*-induced tumorigenesis, the number and size of small intestinal and colonic tumors were examined in all 4 dietary treatment groups. As shown in Fig. 4B, analysis of methylene blue-stained whole mounts of the small intestine revealed a significant reduction (80%, $P < 0.0001$) in tumor numbers in mice maintained for 11 weeks on the MDD diet compared with the MDS diet. Despite this marked suppression in tumor numbers, methyl donor depletion did not affect the size of tumors in the small intestine (data not shown). Consistent with previous studies in *Apc^{Min/+}* mice (24–26), tumors were mainly confined to the small intestine, and their distribution was not affected by dietary methyl donor status. In the colon, although the total number of tumors was not affected by methyl donor depletion (Fig. 4C), there was a trend toward a reduction in the size of colon tumors in the MDD diet group ($P = 0.063$).

As shown in Fig. 4D, there were no medium-sized (between 2 and 4 mm) or large-sized (≥ 4 mm) colon tumors in the MDD group, suggesting that methyl donor deficiency may prevent the progression of colon tumors in *Apc^{Min/+}* mice.

When the start of the MDD diet was delayed until 10 weeks of age, serum folate was reduced by less than 25% in the MDD group ($P < 0.08$) as shown in Fig. 5A. However, cancer suppression was largely lost in the small intestine and colon (Fig. 5B and C). The inhibitory effect on colon tumor growth was also lost (Fig. 5D). The lack of protection, however, may be related to the mild reduction in serum folate levels. These results indicated that the timing and duration of methyl donor depletion is a critical factor in suppression of intestinal tumor formation in the *Apc^{Min/+}* mouse model.

Because we observed extensive weight loss with the MDD diet, we conducted a second study (study 2) using a pair-feeding design (Fig. 1B) to adjust for the potential effect of weight loss on intestinal tumorigenesis. As shown in Fig. 3, pair-fed animals showed the same reduction in body weight gain when compared with the MDD group. However, even after adjusting for reduced body weight gain, the MDD diet induced a striking and significant further tumor reduction ($\sim 96\%$, $P < 0.0001$), as shown in Fig. 6A. Also, as anticipated, the calorie-restricted animals (i.e., MDS pair-fed group) showed a significant reduction ($\sim 54\%$, $P < 0.0001$) in tumor burden (Fig. 6A). In the colon, although the total number of tumors was not affected by methyl donor depletion (Fig. 6B), there was a nonsignificant ($P =$

Figure 5. The effects of methyl donor depletion for 6 weeks on serum folate concentrations and intestinal tumor formation. A, serum folate concentrations were determined by a microtiter plate assay using *Lactobacillus casei*, as described under Materials and Methods; B and C, total number of tumors per mouse in the small intestine (B) and colon (C) from MDS ($n = 5$) and MDD ($n = 8$) mice, respectively. D, colon tumors were classified by size as indicated. Each data point represents an individual mouse and the numbers indicate the mean value for each group. No statistically significant differences were observed between the groups.



0.285) reduction in medium-sized (between 2 and 4 mm) tumors and an absence of large-sized (≥ 4 mm) tumors in the MDD group (Fig. 6C). These data suggested that methyl donor deficiency may prevent the progression of colon tumors in *Apc^{Min/+}* mice, a conclusion that is consistent with our findings in study 1.

Effects of methyl donor status on global gene expression patterns

RNA was isolated from normal-appearing colonic mucosa from study 1 mice maintained on the MDS and MDD diets for 11 weeks. A total of 1,009 genes were differentially expressed between the control and MDD groups at FDR less than 0.1 (refer to Supplementary Data for complete results); 516 genes were upregulated and 493 genes were downregulated. Hierarchical clustering of the most significantly altered genes represented as individual animals is shown in Fig. 7A. Among the significantly differentially regulated genes, many were downregulated and showed distinct clustering patterns (Fig. 7B). Within this set were a number of downregulated genes that are involved in various aspects of immune cell function and inflammation, as well as cell-cycle control.

Validation of differentially expressed genes by qRT-PCR

To provide further validation of the microarray data, we selected 3 differentially regulated genes from the most significantly altered genes identified earlier by *t* tests. Included in this analysis are TNF- α , CXCL1, and CD28. As shown

in Fig. 8, there was a marked downregulation in the expression of TNF- α , CXCL1, and CD28 ($P = 0.0139$, $P = 0.0159$, and $P = 0.0005$, respectively). These data confirmed the gene expression changes identified in Fig. 7B.

Gene set enrichment analysis

To gain additional insight into the molecular alterations that may be associated with methyl donor depletion and tumor suppression, we carried out a GSEA for Gene Ontology categories within the 2 dietary groups. Again, Gene Ontology showed that among the most downregulated categories were those related to immune cell function and inflammatory response. Other downregulated functional categories include cell turnover, comprising the chemokine and cytokine activity, cell-cycle progression, and mitosis. The most upregulated categories were related to extracellular structure and ribosome organization. GSEA for canonical pathway analysis showed that the most downregulated pathways included Toll-like receptor and T-cell receptor signaling, cytokine-cytokine receptor, cell adhesion molecules, and p53 signaling.

During the course of these studies, we began to observe a remarkable overlap in gene expression profiles within the colons of methyl donor-depleted *Apc^{Min/+}* mice and that of a folate depletion study that was recently carried out in human subjects (12). On the basis of these similarities, we compared mouse GSEA data to our recently published human folate depletion study (12). Although we observed significant differences in gene expression patterns in the colons of mice maintained on the MDS and MDD diets, we

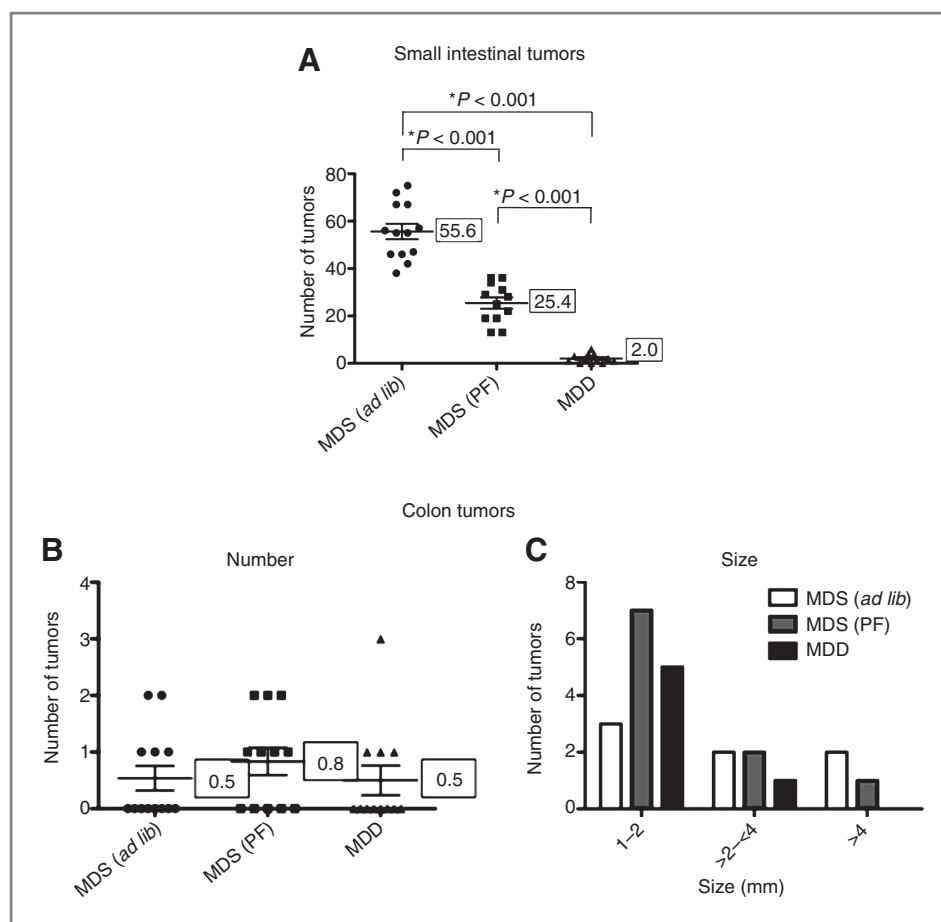


Figure 6. The effect of reduced caloric intake and methyl donor depletion on intestinal tumor formation. A and B, total number of tumors per mouse in the small intestine (A) and colon (B) from MDS *ad libitum* ($n = 13$), MDS pair-fed ($n = 12$) and MDD ($n = 12$) mice, respectively. C, colon tumors were classified by size as indicated. Note that there were no large-sized (≥ 4 mm) colon tumors in the MDD group. Each data point represents an individual mouse and the numbers indicate the mean value for each group. P values are indicated in the Figure as determined using one-way ANOVA followed by Bonferroni's multiple comparison tests.

cannot exclude the possibility that these changes may be due in part to the marked suppression in body weight gain observed in the MDD group. Nevertheless, a direct comparison of GSEA for both Gene Ontology categories and canonical pathways across the 2 species showed that folate and/or methyl donor depletion was associated with a significant downregulation of gene ontology categories and pathways involved in the inflammatory response and mucosal immunity, including components of the innate immune response (Fig. 9).

Discussion

This study shows that dietary restriction of methyl donors, including folate, suppresses intestinal tumorigenesis in *Apc^{Min/+}* mice. Although the tumor suppression is most dramatic in the small intestine, the primary site of tumors in this model, some trend toward a reduction in the size of tumors was also observed in the colon. Importantly, the protective effect of methyl donor depletion is strongly dependent upon the timing and duration of the dietary treatment. Gene expression profiling within the normal colonic mucosa identified a striking downregulation by dietary methyl donor depletion of a large number of genes involved in the immune and inflammatory response. These

observed gene expression changes in mice overlapped extensively with expression changes observed within the human colorectum obtained from subjects maintained on low dietary folate (12), validating the use of this mouse model.

We observed that animals in the methyl-depletion group exhibited a reduced body weight gain during the experimental period. Previous nutritional studies in rodents have also shown that folate deficiency results in a reduction in body weight gain (15, 21–23). There is an extensive body of literature describing the effects of caloric restriction on protection from experimentally induced carcinogenesis, beginning with the seminal studies in the 1940s by Tannenbaum and coworkers (ref. 27; reviewed by Kritchevsky in ref. 28). Thus, we speculated that the tumor-suppressive effects observed in the *Apc^{Min/+}* mice maintained on the MDD diet might be an indirect result of caloric restriction, especially as body weight gain was reduced by almost 40% during the course of treatment in this study. For example, in a recent study in primates, caloric restriction was found to significantly reduce the incidence of age-related diseases, including a suppression of gastrointestinal adenocarcinomas, the most common cancer found in rhesus monkeys, by approximately 50% compared with a control diet (29). In fact, caloric restriction was shown to reduce the frequency of intestinal polyps in *Apc^{Min/+}* mice by almost 60%, an effect

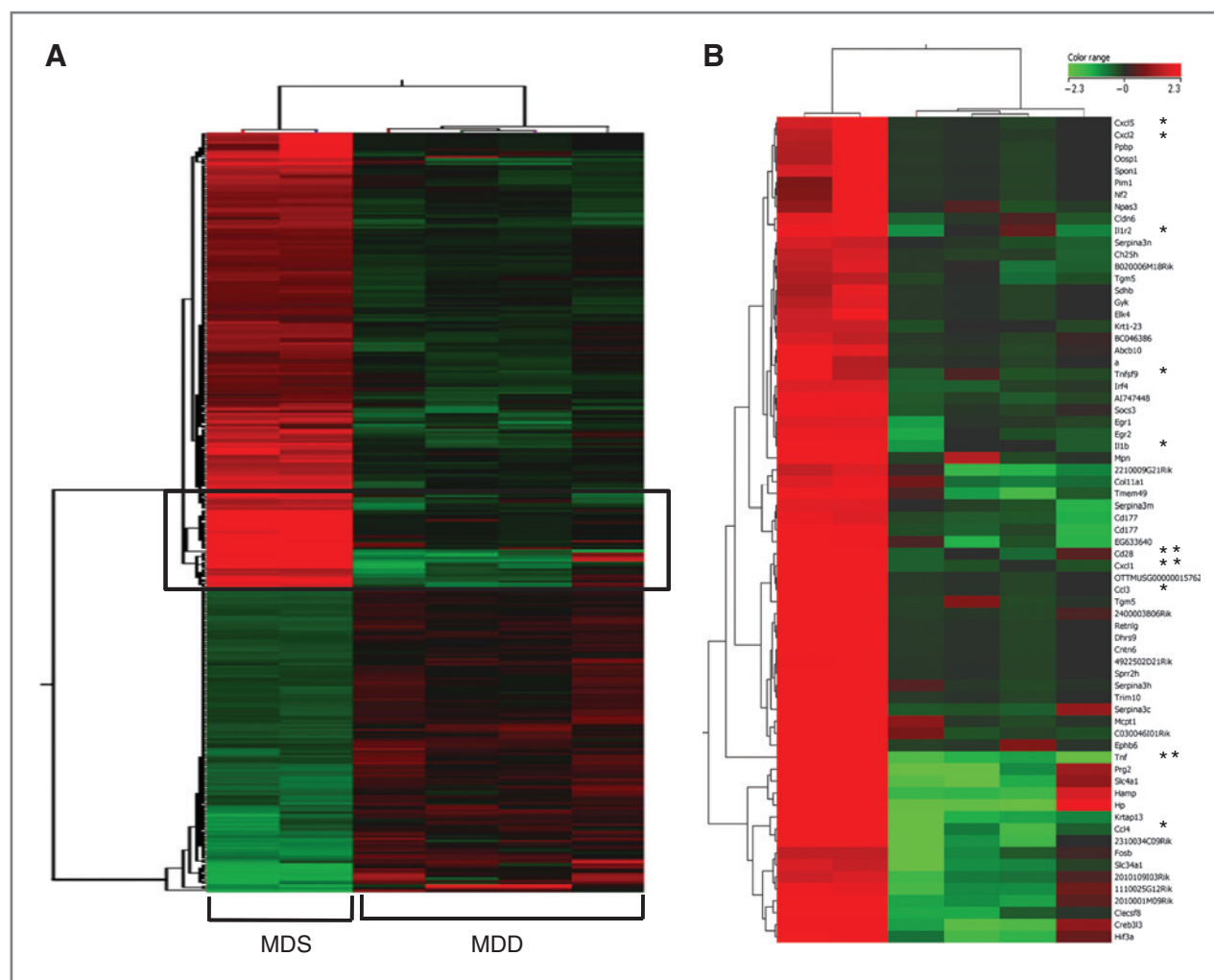


Figure 7. The effect of MDD diet on global gene expression patterns in the colons of *Apc^{Min/+}* mice. A, total RNA was isolated from normal-appearing colonic mucosa from mice maintained on MDS or MDD diets for 11 weeks. Significantly expressed genes between the MDS and MDD groups, identified by adjusted *t* tests, were clustered using a squared Euclidean algorithm. B, a set of genes that are related to inflammation and chemokine signaling are shown enlarged in the right panel. "*" indicates genes related to chemokine pathways and "*" "*" indicates the genes that were further validated by qRT-PCR. The colored scale shown in the Figure represents the change in signal intensities.

that was mediated, in part, through reduced levels of IGF-1 and leptin (30). Caloric restriction may also afford protection against colon carcinogenesis in human populations. In a study of colonic proliferation in obese subjects, caloric restriction significantly reduced markers of colonic proliferation (31). Importantly, these cancer preventive effects are also highly dependent on the extent and duration of caloric restriction, as well as the age at which the restriction was initiated in animal models (32–34). However, in a follow-up pairfeeding study in which *Apc^{Min/+}* mice on MDS diet showed comparable weight loss to the MDD dietary group, we clearly showed that the cancer-suppressive effects of the MDD diet extend beyond the moderate protection observed in the calorie-restricted group.

Therefore, the tumor-suppressive effects observed in this study are unlikely to be due entirely to reduced food intake and the associated reduction in body weight gain.

Furthermore, when methyl donor deficiency was delayed until 10 weeks of age, mice continued to lose weight (up to 40% at the end of the study) as shown in Supplementary Fig. S1, yet the cancer protection was essentially lost. In addition, low serum folate levels were comparable in both study groups. Moreover, the colons of methyl deficient animals exhibited gene expression profiles resembling published human data, yet human subjects were under strict dietary control and did not lose weight during the 3-month study (12). Thus these data indicate that the tumor-suppressive effect is most likely related to the timing and extent of methyl donor deficiency rather than simply caloric restriction.

In previous studies, the timing of folate intervention was also found to influence the number of intestinal tumors in *Apc^{Min/+}* mice. When moderate folate supplementation was started before the formation of neoplastic foci in 3-week-old

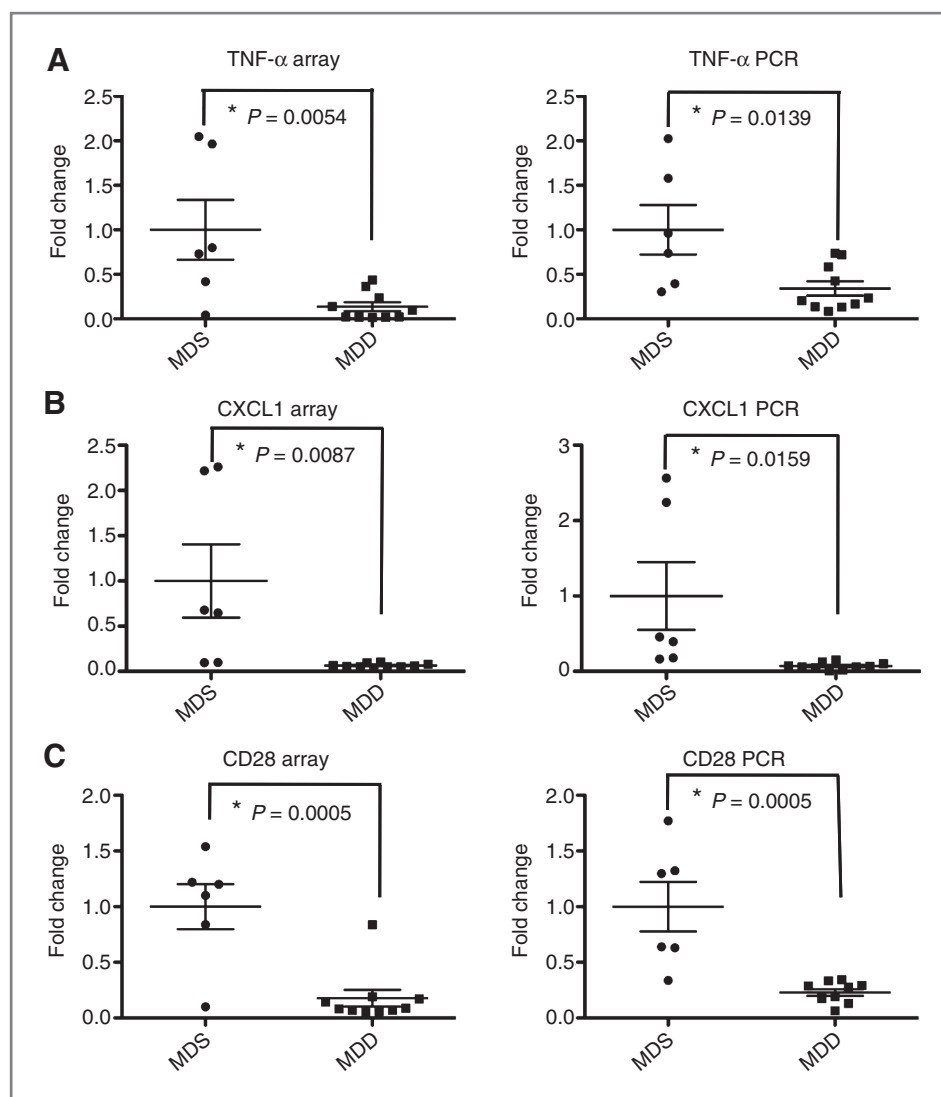


Figure 8. Validation of gene expression changes induced by the MDD diet in *Apc*^{Min/+} colons. Total RNA was isolated from the colons of *Apc*^{Min/+} mice as described under Materials and Methods. To validate the array data obtained from Groups I and II, real-time PCR analysis was carried out on 3 of the most significantly downregulated genes within the proinflammatory network, including TNF- α (A), CXCL1 (B), and CD28 (C). Each data point represents the expression fold-change value for an individual mouse, with *P* values as indicated in the Figure. The left panels show the microarray data and the right panels show the qRT-PCR data.

mice, there was a significant decrease in the number of intestinal and colonic tumors (35). However, when folate levels were moderately reduced in 6-week-old mice after neoplastic foci have developed, only small intestinal adenomas were reduced (35). The same group also reported that dietary folate supplementation at weaning suppresses both ileal and colonic tumor formation (24). This study shows that methyl donor depletion suppresses tumor formation when the dietary intervention was initiated at 5 weeks of age, at just about the time when neoplastic foci are established in this model (35). In contrast to a previous study (35), however, the suppressive effect was lost when the MDD intervention was initiated at 10 weeks of age after establishment of neoplastic foci. In addition, Lindzon and colleagues (36) tested the effect of folate supplementation in an azoxymethane-treated rat model. Supplementation with folic acid beginning 6 weeks after carcinogen treatment was found to promote the growth of preneoplastic aberrant crypt foci and tumors (36).

In this study, we describe significant alterations occurring within a number of signaling networks associated with the colonic mucosa in *Apc*^{Min/+} mice that result from methyl donor deficiency. Using GSEA, we show that several of the most significantly enriched gene categories are related to immune and inflammatory response. Within these categories of inflammatory genes showing significant downregulation are a large number of cytokines and chemokines (Fig. 7B). Canonical pathway analyses showed that chemokine receptor signaling and innate and mucosal immune responses were also downregulated, in remarkable agreement with our recent human study in which dietary folate levels were reduced for up to 8 weeks (Fig. 9; ref. 12). In the human study, folate depletion also resulted in reduced expression of a number of immune response genes, including, most notably, cytokines and chemokines (12). Interestingly, folate supplementation in the same human study increased the expression of inflammatory and immune response genes

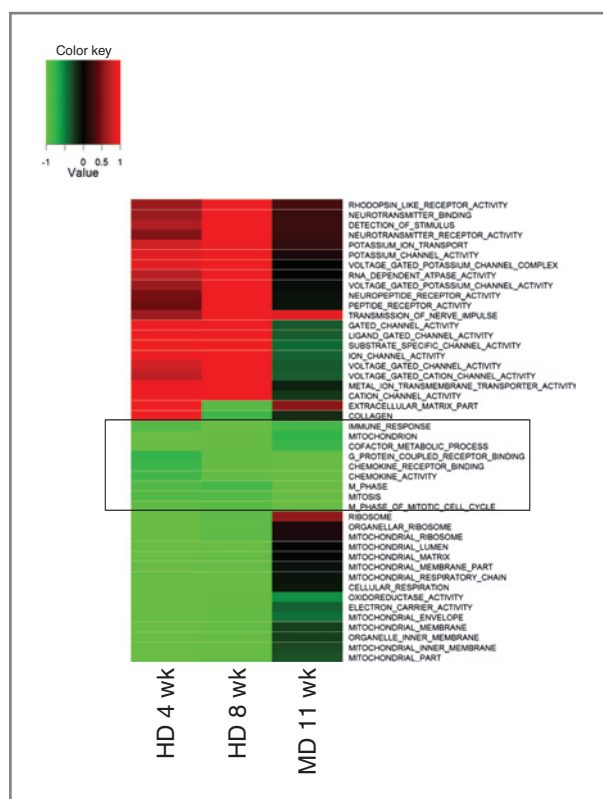


Figure 9. Heat map of Gene Set Enrichment Analysis for Gene Ontology comparing folate/MDD mice to a human folate depletion study. The figure compares significantly enriched GO categories in the human 4-week (HD 4 weeks) and 8-week (HD 8 weeks) dietary folate depletion studies to the present 11-week folate/methyl donor depletion study. Only categories with an FDR Q value of less than 0.001 in at least one condition are shown. Colors indicate downregulation (green) or upregulation (red), and values are represented as $1 - P$ (with downregulation assigned negative values). Note that gene categories related to immune function and inflammation are downregulated by folate/methyl depletion in the mouse colon and closely resemble the human data.

(12). In a related human study, the administration of 1.2 mg folic acid for 12 weeks increased the levels of serum proteins involved in the regulation and activation of immune function such as acute phase reactants and complement fixation (13). The analysis of circulating inflammatory markers obtained from the human Aspirin/Folate Polyp Prevention trial suggested that folic acid administration antagonizes the colon cancer-suppressive effects of aspirin (14). Taken together, these studies suggested that folate/methyl donor status may modulate the intestinal inflammatory response.

Although we found that methyl donor deficiency reduced the expression of a panel of genes associated with the immune response, the impact of these expression changes on the inflammatory response within the colonic mucosa is not known at the present time. There is abundant evidence supporting the association of chronic inflammation and colon cancer risk (37), and numerous inflammatory signals are important modulators of colon

cancer risk. In fact, the suppression of intestinal inflammation in *Apc^{Min/+}* mice has already been shown to reduce tumor burden (26, 38–40). In addition, the use of anti-inflammatory agents such as NSAIDs can also reduce this risk, but the significant potential for serious adverse effects limit the long-term use of these agents for chemoprevention (41–43). Our mouse data clearly show that folate/methyl donor depletion does in fact markedly reduce tumor incidence in a high-risk animal model, but further studies are necessary to more definitely establish the implications of this dietary intervention on the human colonic mucosa.

In summary, this study addresses the influence of folate/methyl donor status on intestinal tumorigenesis. We show that this dietary restriction suppresses intestinal tumorigenesis in *Apc^{Min/+}* mice. Although the tumor suppression is most dramatic in the small intestine, the primary site of tumor formation in this model, a trend toward tumor protection was also observed in the colon. Importantly, the protective effect of methyl donor depletion is strongly dependent upon the timing and duration of the dietary intervention. Gene expression profiling within the normal colonic mucosa identified a striking downregulation by dietary methyl donor depletion of a large number of genes involved in the immune and inflammatory response. These gene expression changes in mice generally resemble the results of our human folate depletion study (12), validating the use of this mouse model to study the interplay between dietary interventions and colon cancer risk.

Disclosure of Potential Conflicts of Interest

E.J. Greenspan has held the title of program manager in NIH/National Cancer Institute.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Kadaveru, P. Protiva, E.J. Greenspan, Y.-I. Kim, D.W. Rosenberg

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Additional microarray supporting material could be obtained on request from Petr Protiva (E-mail: Petr.Protiva@yale.edu).

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Dietary Methyl Donor Depletion Protects Against Intestinal Tumorigenesis in *Apc^{Min/+}* Mice

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