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Chemopreventive Effects of Dietary Folate on Intestinal Polyps in *Apc*+/− *Msh2*−/− Mice[1]

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ABSTRACT

Epidemiological and animal studies (reviewed in Y. I. Kim, J. Nutr. Biochemistry, *10:* 66–88, 1999; J. B. Mason and T. Levesque, Oncology, *10:* 1727–1743, 1996) suggest that dietary folate intake is inversely related to the risk of colorectal cancer. However, the optimal timing of folate intervention and mechanisms by which folate modulates colorectal carcinogenesis have not been clearly established. A recently developed murine model of intestinal tumorigenesis, which carries a heterozygous mutation in the *Apc* gene and a null mutation in the *Msh2* gene (*Apc*+/−*Msh2*−/−), was used to determine the effect of dietary folate on intestinal tumorigenesis. *Apc*+/− *Msh2*−/− mice were randomized to receive either 0 or 8 mg of folate/kg diet starting at either 3 or 6 weeks of age. The 3- and 6-week diet starts represent intervention before and after the establishment of neoplastic foci, respectively. At 11 weeks of age, mice were killed, and the small intestines and colons were analyzed for adenomas and aberrant crypt foci (ACF). Serum folate concentrations were determined by a standard microbiological assay. Genomic DNA methylation was assessed by *in vitro* [^{3}H]methyl incorporation into hepatic DNA and by a methyl-sensitive restriction digestion method. Microsatellite instability was determined in matched normal and polyp DNA from the small intestine and colon at 5 loci. Serum folate concentrations accurately reflected dietary folate levels ($P < 0.005$). Folate supplementation, started before the establishment of neoplastic foci, significantly decreased the number of small intestinal adenomas (by 2.7-fold; $P = 0.004$) and colonic ACF (by 2.8-fold; $P = 0.028$) and colonic adenomas (by 2.8-fold; $P = 0.1$) compared with a moderate degree of folate deficiency. In contrast, a moderately folate-deficient diet, started after the establishment of neoplastic foci, significantly reduced the number of small intestinal adenomas (by 4.2-fold; $P = 0.001$) but had no effect on colonic ACF and adenomas compared with folate supplementation. Genomic DNA methylation and microsatellite instability do not seem to play a major role in folate-modulated intestinal and colonic tumorigenesis in this model. In conclusion, in this murine model, dietary folate supplementation significantly protects against small intestinal and colorectal tumorigenesis if it is provided before the establishment of neoplastic foci However, if it is provided after the establishment of neoplastic foci, dietary folate seems to have an opposite effect. These data suggest that the timing of folate intervention is critical in providing an effective and safe chemopreventive effect on intestinal tumorigenesis. Notwithstanding the limitations associated with this model, our data suggest that the optimal timing of folate intervention must be established before folate supplementation can be used as a safe chemopreventive agent against colorectal cancer.

INTRODUCTION

Folate has recently emerged as an important nutritional factor that may modulate colorectal carcinogenesis (reviewed in Refs. 1 and 2). The majority of published epidemiological studies indicate that dietary folate intake is inversely associated with the risk of developing colorectal adenomas (3–8) and cancers (7, 9–17) in a dose-dependent fashion. Collectively, these studies suggest an approximately 40% reduction in the risk of colorectal neoplasms in individuals taking the highest amount of folate compared with those taking the lowest (3–17). In addition, studies conducted in individuals with ulcerative colitis, a disease associated with an enhanced risk of both folate deficiency and colorectal neoplasia (18), have corroborated the inverse association between folate status and colorectal cancer risk observed in population-based epidemiological studies (19–21).

Two animal studies, performed in the dimethylhydrazine colorectal cancer rat model, have supported a causal relationship between folate deficiency and colorectal cancer (22, 23). These studies have also shown a dose-dependent protective effect of modest levels of dietary folate supplementation up to four times the basal dietary requirement (22, 23). Levels of dietary folate greater than four times the dietary requirement did not convey additional benefits; in fact, there was a nonsignificant trend toward increased colorectal tumorigenesis in rats fed a supraphysiological dose of folate (20 times the daily requirement; Ref. 23). This study suggests that supplemental folate may have two distinct actions in this model. At modest levels of supplementation beyond the dietary requirement, folate seems to possess an inhibitory effect on the genesis and progression of colorectal neoplasms (22, 23). However, exceptionally high supplemental folate levels may promote the progression of chemically induced colorectal neoplastic foci (23). In support of this latter finding, dietary folate supplementation exceeding the basal requirement by 1000 times increased the development of ACF,[3] the probable earliest precursor of colorectal cancer (24), compared with a control diet in another rat study in which azoxymethane (a metabolite of dimethylhydrazine) was used (25).

Although some similarities do exist, tumor development in chemical rodent models of colon cancer differs in several important respects from that observed in humans (26, 27). The chemically induced carcinomas often arise from flat foci of dysplasia rather than from adenomatous polyps. The relatively high doses of genotoxic chemical carcinogens differ from the natural etiological causes involved in most cases of sporadic colorectal cancer in humans. Most importantly, molecular genetics of chemical rodent models are significantly different from those observed in human colorectal carcinogenesis. For example, although K-*ras* mutations are as frequently mutated in these rodent models as in human colorectal cancer (28, 29), the *Apc* and *p53* genes, two commonly mutated genes in human colorectal cancer (30), are either mutated to a much lesser extent or not mutated at all in these rodent models (31–35). Also, recent evidence suggests that chemi-

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[3] The abbreviations used are: ACF, aberrant crypt foci; CpG, cytosine-guanine (dinucleotides); HNPCC, hereditary nonpolyposis colorectal cancer; SAM, *S*-adenosylmethionine.

cally induced colorectal carcinogenesis in rodents involves alterations in unique colon cancer susceptibility genes that are not implicated in human colorectal cancer (36, 37).

Recently developed genetic murine models characterized by the spontaneous development of small intestinal and colonic tumors have provided an excellent opportunity to investigate the effects of environmental and genetic factors in both familial and sporadic colorectal carcinogenesis. One such model, *Apc*+/− *Msh2*−/−, was generated by crossing Min (multiple intestinal neoplasia; *Apc*+/−) mice with *Msh2*−/− mice (38). Min mice carry a heterozygous germ-line mutation at codon 850 of the mouse *Apc* gene and develop approximately 25–75 small intestinal adenomas and 1–5 colorectal adenomas by 160–180 days, at which time they become moribund and die from anemia and intestinal obstruction (39, 40). The wild-type *Apc* allele is lost in polyps from Min mice (41), and, therefore, this model resembles the syndrome of familial adenomatous polyposis coli in humans (42). The *MSH2* gene is one of several mismatch repair genes that ensure accurate replication of the genome during cell division (43). Germ-line mutations in the mismatch repair genes have been implicated in HNPCC and somatic mutations in about 15% of sporadic colorectal cancer (43). Mutations in the mismatch repair genes result in a "mutator phenotype," in which loss of postreplicative DNA repair increases the mutation rate, and results in a replication error phenotype or microsatellite instability (43).

Apc+/− *Msh2*−/−mice display an accelerated intestinal adenoma phenotype and develop numerous dysplastic colonic ACF (38). They develop approximately 350 small intestinal tumors, 8 colon tumors, and 55 ACF by 80 days of age, at which time they become moribund and die of anemia or bowel obstruction (38). The average time required for a nascent tumor to develop into a macroscopically visible adenoma in these mice was estimated to be 42 and 27 days in the small intestine and colon, respectively (38). The *Apc*+/− *Msh2*−/− mouse model provides many advantages compared with previous animal models of colorectal neoplasia. These mice develop intestinal neoplasms spontaneously without the need for carcinogens. In particular, in contrast to other genetic models of colorectal cancer, *Apc*+/− *Msh2*−/− mice develop colonic ACF, the probable earliest precursor of colorectal cancer observed in humans (24). This allows this model to be used in studies examining early events of colorectal neoplasia. The *Apc*+/− *Msh2*−/− mouse model is phenotypically and genotypically more similar to human colorectal cancer, thereby making this model more clinically relevant than other available animal models. Because of the accelerated nature of tumorigenesis in this model, it is possible to test the effects of chemopreventive agents on initiation and progression of small intestinal and colorectal polyps and ACF in a relatively short time.

This study investigated the effects of dietary folate on the initiation and promotion of intestinal tumorigenesis in *Apc*+/−*Msh2*−/− mice. Furthermore, possible mechanisms by which dietary folate can modulate small intestinal and colorectal tumorigenesis in this model were investigated.

MATERIALS AND METHODS

This study was approved by the Animal Care Committee of the Samuel Lunenfeld Research Institute.

Mice. Min (*Apc* +/−*Msh2*+/+) mice were bred at the Samuel Lunenfeld Research Institute on the C57BL/6J strain (original breeding pair from The Jackson Laboratory, Bar Harbor, ME). The generation of *Msh2*−/− mice has been described previously (44, 45). *Apc* +/−*Msh2*+/− mice were generated by crossing male Min mice with female *Msh2*+/− mice. Male *Apc*+/− *Msh2*+/− mice were then crossed with female *Msh2*+/− mice to generate *Apc*+/− *Msh2*−/− mice as described previously (38).

Genotyping. Ear punch tissue was processed for genotyping using PCR-based assays to determine *Apc* and *Msh2* status as described previously (38, 44).

Diet. Mice were fed an amino-acid-defined diet containing either 0 or 8 mg folate/kg diet (Dyets, Bethlehem, PA; Ref. 46). These diets constitute standard means of inducing folate deficiency or providing supplemental dietary folate in rodents (46). The diet containing 0 mg folate/kg produces progressive folate deficiency of a moderate degree without anemia, growth retardation, or premature death through week 5, after which systemic folate indicators stabilize (22). Although this diet is completely devoid of folate, intestinal microflora are capable of *de novo* synthesis of folate, some of which is incorporated into the tissue folate of the host; this prevents severe folate deficiency (47). The degree of folate deficiency produced by this diet is comparable with that associated with the increased risk of colorectal cancer in humans (3–17, 19–21). The diet containing 8 mg folate/kg represents folate supplement 4 times the basal dietary requirement for rodents (*i.e.*, 2 mg folate/kg; Ref. 48). This level of folate was chosen because the 8 mg/kg level has consistently provided a degree of chemoprevention against colorectal cancer in previous rodent studies (22, 23). These diets contained 50 g cellulose/kg, 60% calories as carbohydrates, 23% fat, and 17% L-amino acid. The amount of methyl donors, methionine, choline and vitamin B_{12}, were 8.2 g, 2.0 g and 50 μg per kg diet, respectively. Diet and water were provided *ad libitum*. Detailed composition of the diets is shown in Tables 1 and 2.

Dietary Intervention. Mice were randomized to receive the amino-acid-defined diet containing either 0 or 8 mg/kg folate starting at either 3 or 6 weeks of age. Mice were then killed at 11 weeks of age. Given that the average time required for a nascent tumor to develop into a macroscopically visible adenoma in these mice was estimated to be 42 and 27 days in the small intestine and colon, respectively (38), the 3-week time point was chosen to represent a time which was before the establishment of most macroscopic neoplastic foci. The 6-week time point was chosen to represent the time after the establishment of neoplastic foci. There were 7 mice in each diet group at the 3-week-diet start; and 10 and 7 mice in the 0- and 8-mg folate/kg diets, respectively, in the 6-week-diet start.

Enumeration of Small Intestinal Adenomas, Colonic ACF, and Tumors. Mice were killed by cervical dislocation. Intestines were immediately removed and flushed with Krebs buffer solution to remove fecal debris. The entire length of the small intestine and colon was opened longitudinally, laid flat on Whatman filter paper, and fixed for at least 3 h in 10% neutral buffered formalin. The mucosa of the fixed small intestine and colon was stained with methylene blue and examined in a blinded fashion for tumors and ACF by gross inspection and light microscopy as described previously (49). All of the small intestine and colon tumors and colonic ACF were counted in a blinded fashion. Previous studies have shown that all small intestinal tumors are adenomas and the majority of colonic ACF are dysplastic in this model (38). Representative small bowel tumors (adenomas) and all of the colonic tumors were processed in a standard manner for H&E staining and histologically analyzed by a gastrointestinal pathologist [A. M.] blinded to the study groups.

Folate Concentration Determination. At the time of death, blood was withdrawn from the heart into vacutainer tubes containing EDTA using a preheparinized 18-gauge needle and was centrifuged at 800 × *g* for 10 min at 4°C. Serum was stored at −70°C in 0.5% ascorbic acid for the serum folate assay. Serum folate concentrations were measured by a microtiter plate assay using *Lactobacillus casei* as described previously (50).

Table 1 *Diet composition*

Nutrients (g/kg diet)	Deficient	Supplemented
Total L-amino acids[a]	171.44	171.44
Mineral mix[b]	57.96	57.96
Vitamin mix[c]	10.00	10.00
Dextran	407.00	406.00
Sucrose	195.00	194.40
Cellulose	50.00	50.00
Corn oil (with 0.015% BHT)[d]	100.00	100.00
Choline chloride	2.00	2.00
Sodium bicarbonate	6.60	6.60
Folic acid-sucrose mix (5 mg folic acid/g)	0.00	1.60
Total	1000.00	1000.00

[a] See Table 2, column 1.
[b] See Table 2, column 2.
[c] See Table 2, column 3.
[d] BHT, butylated hydroxytoluene.

Table 2 *Composition of L-amino acids and of mineral and vitamin mixes*

L-Amino acids (g/kg diet)		Minerals (g/kg diet)		Vitamins (g/kg diet)	
		Calcium carbonate	14.60000	Thiamin HCl	0.006
L-Alanine	3.50	Calcium phosphate, dibasic	0.17000	Riboflavin	0.006
L-Arginine (free base)	11.20	Sodium chloride	12.37000	Pyridoxine HCl	0.007
L-Asparaginine	6.00	Potassium phosphate, dibasic	17.16000	Nicotinic acid	0.030
L-Aspartic acid	3.50	Magnesium sulfate, anhydrous	2.45000	Calcium pantothenate	0.016
L-Cystine	3.50	Manganese sulfate, monohydrate	0.18000	Cyanocobalamin	0.00005
L-Glutamic acid	35.00	Ferric citrate	0.62000	Vitamin A palmitate (500,000 IU/g)	0.008
Glycine	23.30	Zinc carbonate	0.05700		
L-Histidine (free base)	3.30	Cupric carbonate	0.05400	Vitamin D3 (400,000 IU/g)	0.0025
L-Isoleucine	8.20	Potassium iodide	0.00058	Vitamin E acetate (500 IU/g)	0.100
L-Leucine	11.10	Sodium selenite	0.00058	Menadioine sodium bisulfite	0.00080
L-Lysine	14.40	Chromium potassium sulfate	0.01900	Biotin	0.00002
L-Methionine	8.20	Sodium fluoride	0.00230	Sucrose	9.82363
L-Phenylalanine	11.60	Molybdic acid, ammonium salt	0.00120		
L-Proline	3.50	Sucrose	10.27534		
L-Serine	3.50				
L-Threonine	8.20				
L-Tryptophan	1.74				
L-Tyrosine	3.50				
L-Valine	8.20				
Total	171.44		57.96		10.00

DNA Extraction. Areas corresponding to microscopically confirmed small intestinal and colonic adenomas on H&E staining were marked on matched paraffin blocks. DNA from adenomas was extracted as crude preparations from paraffin blocks using proteinase K lysis mix [10 mM Tris-HCl (pH 8.0), 100 mM KCl, 2.5 mM $MgCl_2$, 0.45% Tween 20, and 1 mg/ml proteinase K] as previously described (45). Care was taken to avoid contamination from adjacent nonneoplastic tissues. The sections were homogenized in the lysis mix and digested for 1 h at 65°C followed by 10 min at 95°C. Extracted DNA was stored at −20°C until DNA methylation and microsatellite assays. Small intestinal and colonic DNA from normal mucosa was extracted from areas corresponding to normal histology from paraffin blocks in a similar fashion (45). DNA from the liver, snap-frozen at the time of sacrifice and stored at −70°C, was extracted by a standard technique using a lysis buffer containing proteinase K followed by phenol, chloroform, and isoamyl alcohol organic extraction (51).

Genomic DNA Methylation Determination. The methylation status of CpG sites in genomic DNA from the liver was determined by the *in vitro* methyl acceptance capacity of DNA using [^{3}H-methyl]SAM as a methyl donor and a prokaryotic CpG DNA methyltransferase, Sss1, as previously described (52). The manner in which this assay is performed produces a reciprocal relationship between the endogenous DNA methylation status and the exogenous [^{3}H]methyl incorporation. Briefly, 500 ng of liver DNA were incubated with 2 μCi [^{3}H-methyl]SAM (New England Nuclear, Boston, MA), 3 units Sss1 methylase (New England Biolabs, Beverly, MA), 1× Sss1 buffer [120 mM NaCl, 10 mM Tris-HCl (pH 7.9), 10 mM EDTA, and 1 mM DTT] in a total volume of 30 μl at 30°C for 1 h. The *in vitro* methylated DNA was isolated from a 15-μl aliquot of the reaction mixture by filtration on a Whatman DE-81 ion exchange paper (Fisher Scientific, Springfield, NJ). The DNA was washed three times with 20 ml of 0.5 M sodium phosphate buffer (pH 7.0), followed by 2 ml of 70% ethanol and 2 ml of absolute ethanol. The filters were air-dried and the resulting radioactivity of the DNA retained in the filters was measured by scintillation counting using a nonaqueous scintillation fluor. The amount of radiolabel bound to a filter from an incubation mixture lacking Sss1 was used as background and was subtracted from the values obtained with mixtures containing DNA. The background value was always <1% of the uptake observed with DNA samples. All of the analyses were performed in duplicate.

Genomic DNA methylation status assessed by the *in vitro* methyl acceptance capacity of DNA was confirmed by a methyl-sensitive restriction digestion method. Three samples from folate-depleted and -supplemented groups at 3 and 6 week diet starts were selected as representatives of their respective groups because their serum folate concentrations were closest to the respective means of their groups. Genomic liver DNA was digested with *Hpa*II (New England Biolabs) overnight at a final concentration of 20 units/μg DNA at 37°C in a buffer provided by the supplier. One additional DNA sample from each group was selected and digested with *Msp*I (New England Biolabs). *Hpa*II and *Msp*I are isoschizomers that cleave the sequence 5′-CCGG-3′ between the two cytosine residues. *Hpa*II is unable to cleave CCGG if the internal cytosine is methylated whereas *Msp*I can cleave CCGG regardless of the methylation of the internal cytosine residue (53). A *Hpa*II specific oligonucleotide (5′-TAT AGC CGG CTA TA-3′) was added in 10-fold molar excess of genomic DNA to increase the cutting efficiency of *Hpa*II (54). Digested DNA (150 ng/lane) was separated on a 0.8% agarose gel and transferred to a nylon membrane using a Southern blot technique under standard conditions (55). A mouse centromeric minor satellite repeat sequence derived from plasmid MR150 (generously provided by Dr. Janet Rossant, Samuel Lunenfeld Research Institute, Toronto, Ontario, Canada) was used to hybridize restricted DNA fragments as previously described (56). pMR150 was labeled with digoxigenin-11-dUTP using the PCR DIG Probe Synthesis kit (Boehringer Mannheim, Montreal, Quebec, Canada) and hybridized with the membrane that was subsequently exposed according to the manufacturer's protocol.

Microsatellite Instability Assay. Microsatellite instability was detected by comparison of electrophoretic mobility of amplified normal and neoplastic DNA using primers from five loci on mouse chromosomes 1 (*D1 Mit4*), 2 (*D2 Mit16*), 5 (*D5 Mit10*), 6 (*D6 Mit8*), and 10 (*D10 Mit2*) as described previously (Research Genetics, Huntsville, AL; Refs. 38, 45, 46). Matched normal and neoplastic DNA from the small intestine and colon were selected from each dietary group at two time points. Primer labeling and PCR amplification were performed according to the protocol supplied by Research Genetics for Mouse Map Pairs with the following modification. A single primer (10–15 pmol) from each primer pair was end-labeled using T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]dATP (New England Nuclear) according to Sambrook (55). A 4-μl aliquot of the PCR products was mixed with formaldehyde dye mix (2 μl), denatured at 85°C for 2 min and electrophoresed on 7% polyacrylamide gels under denaturing conditions for 2–3 h. Gels were dried and exposed to X-ray film for 12–72 h. A positive case was defined as one showing instability at one or more loci, confirmed in two independently performed PCR reactions.

Statistics. The distribution of each variable was assessed graphically to determine whether it was normally distributed. Those variables that were not normally distributed were subjected to logarithmic transformation before performing a significance test. Comparisons of means between the folate-depleted and -supplemented groups were assessed by Student's *t* test. Statistical analyses were performed using SYSTAT 5 for Macintosh (Systat, Evanston, IL). All of the significance tests were two-sided, and were considered statistically significant if the observed significance level was less than 0.05. Results are expressed as mean ± SE of the untransformed data.

RESULTS

Effects of Dietary Folate Deficiency and Supplementation Beginning at 3 Weeks of Age (*i.e.*, Before the Establishment of Neoplastic Foci) on Intestinal Tumorigenesis

Body Weight and Average Daily Food Consumption. Although the mean weight of the folate-deficient mice was 8–18% lower than

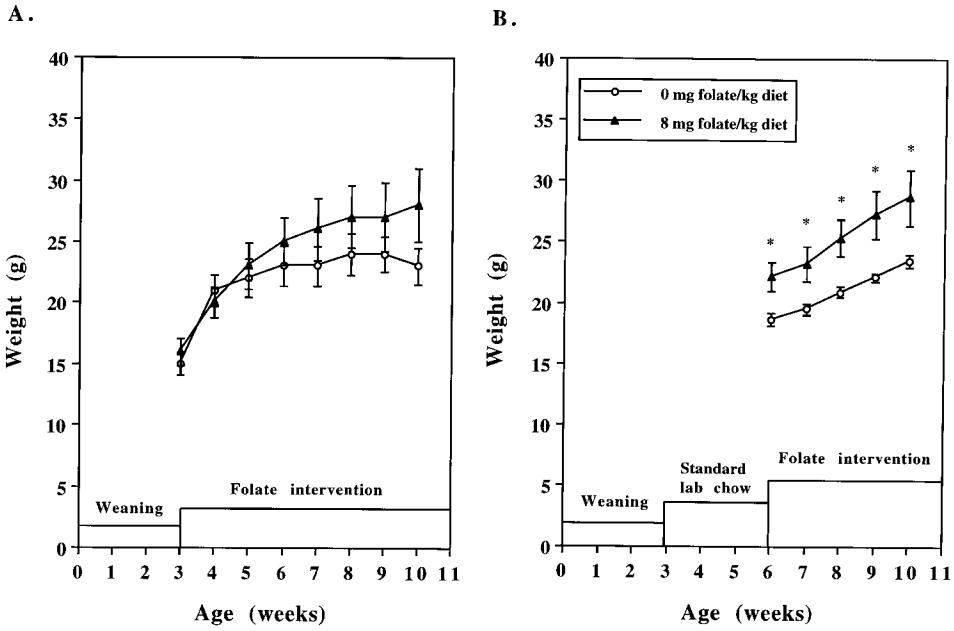

Fig. 1. Average body weights of animal groups as a function of age. *A*, in study 1, mice ($n = 7$ in each group) were randomized to receive the amino-acid-defined diet containing either 0 or 8 mg folate/kg diet for 8 weeks starting at 3 weeks of age (*i.e.*, before the establishment of neoplastic foci). *B*, in study 2, mice ($n = 7$ and 10, respectively) were randomized to receive the amino-acid-defined diet containing either 0 or 8 mg folate/kg diet for 5 weeks starting at 6 weeks of age (*i.e.*, after the establishment of neoplastic foci). *, $P < 0.03$ compared with the 0 mg folate/kg group at each time point.

the folate-supplemented mice during weeks 3 through 8 of dietary intervention, the difference was not statistically significant between these groups (Fig. 1*A*). No premature deaths occurred. This finding indicates that folate deficiency in the mice that were fed 0 mg folate/kg was moderate, otherwise severe growth retardation or premature death would have occurred (57). The average daily food consumption, which was determined on a preassigned day of each week, was also similar between the two groups (data not shown).

Serum Folate Concentrations. The mean serum folate concentration was 4.2-fold lower in mice fed the folate-depleted diet than those on the supplemented diet ($P < 0.001$, Table 3). The mean serum folate concentrations of the folate-depleted and -supplemented mice were comparable with those observed in rats placed on similar diets (22, 23).

Development of Small Intestinal Adenomas and Colonic ACF and Adenomas. Mice fed the folate-supplemented diet had a 2.7-fold lower number of small intestinal adenomas compared with those fed the folate-depleted diet ($P = 0.004$; Table 3). This protective effect of dietary folate supplementation on the development of small intestinal adenomas was consistently observed in the duodenum, jejunum, and ileum ($P < 0.02$, Table 3).

In the colon, the folate-supplemented mice developed significantly fewer ACF (by 2.8-fold) compared with the folate-deficient mice ($P = 0.028$; Table 3). Although folate-supplemented mice had a 2.8-fold lower incidence of colonic adenomas compared with the folate-depleted mice, this fell short of statistical significance ($P = 0.1$; Table 3).

Genomic DNA Methylation Status. The extent of genomic DNA methylation was determined in the liver. There was no difference in the extent of genomic DNA methylation as assessed by the *in vitro* methyl acceptance capacity of DNA and a methyl-sensitive restriction digestion method between the folate-depleted and -supplemented groups (Fig. 2, *A* and *C*). These data suggest that a moderate degree of folate deficiency of 8 weeks duration did not result in a significant degree of genomic DNA hypomethylation.

Microsatellite Instability. A total of 22 small intestinal and colonic adenomas from 12 mice were analyzed for microsatellite instability. No difference in electrophoretic mobility was observed between normal and tumor tissue from either of the diet groups at the five loci tested (Fig. 3*A*). These data suggest that widespread microsatellite instability is probably not a major factor leading to accelerated small intestinal and colonic adenomas in these mice. These data also suggest that superimposed folate deficiency and supplementation had no significant observable effect on microsatellite instability.

Table 3 *Effects of dietary folate intervention on serum folate concentrations, small intestinal and colonic adenomas, and colonic ACF*[a]

Age at diet start	Folate intervention before the establishment of neoplastic foci			Folate intervention after the establishment of neoplastic foci		
	3 weeks			6 weeks		
	Deficient	Supplemented	*P*	Deficient	Supplemented	*P*
Dietary folate (mg folate/kg diet)	0 ($n = 7$)	8 ($n = 7$)		0 ($n = 10$)	8 ($n = 7$)	
Duration of folate intervention (weeks)	8	8		5	5	
Serum folate (ng/ml)	15.0 ± 2.6	63.5 ± 7.7	<0.001	19.8 ± 3.2	95.6 ± 27.0	0.004
Average number of total small intestinal adenomas per mouse	299.4 ± 27.4	110.3 ± 45.2	0.004	70.0 ± 17.1	295.3 ± 59.6	0.001
Duodenal adenomas	75.8 ± 9.6	27.3 ± 9.2	0.003	25.9 ± 7.2	63.4 ± 12.2	0.013
Jejunal adenomas	108.4 ± 15.3	36.0 ± 15.5	0.006	20.1 ± 4.8	124.7 ± 34.2	0.002
Ileal adenomas	115.1 ± 10.5	47.0 ± 21.0	0.013	24.0 ± 6.4	107.1 ± 20.2	<0.001
Average number of colonic adenomas per mouse	1.7 ± 0.5	0.6 ± 0.4	0.1	2.4 ± 1.2	2.4 ± 1.0	0.99
Average number of colonic ACF per mouse	55.1 ± 12.3	19.4 ± 7.3	0.028	34.5 ± 12.0	42.4 ± 7.9	0.63

[a] Values are means ± SE.

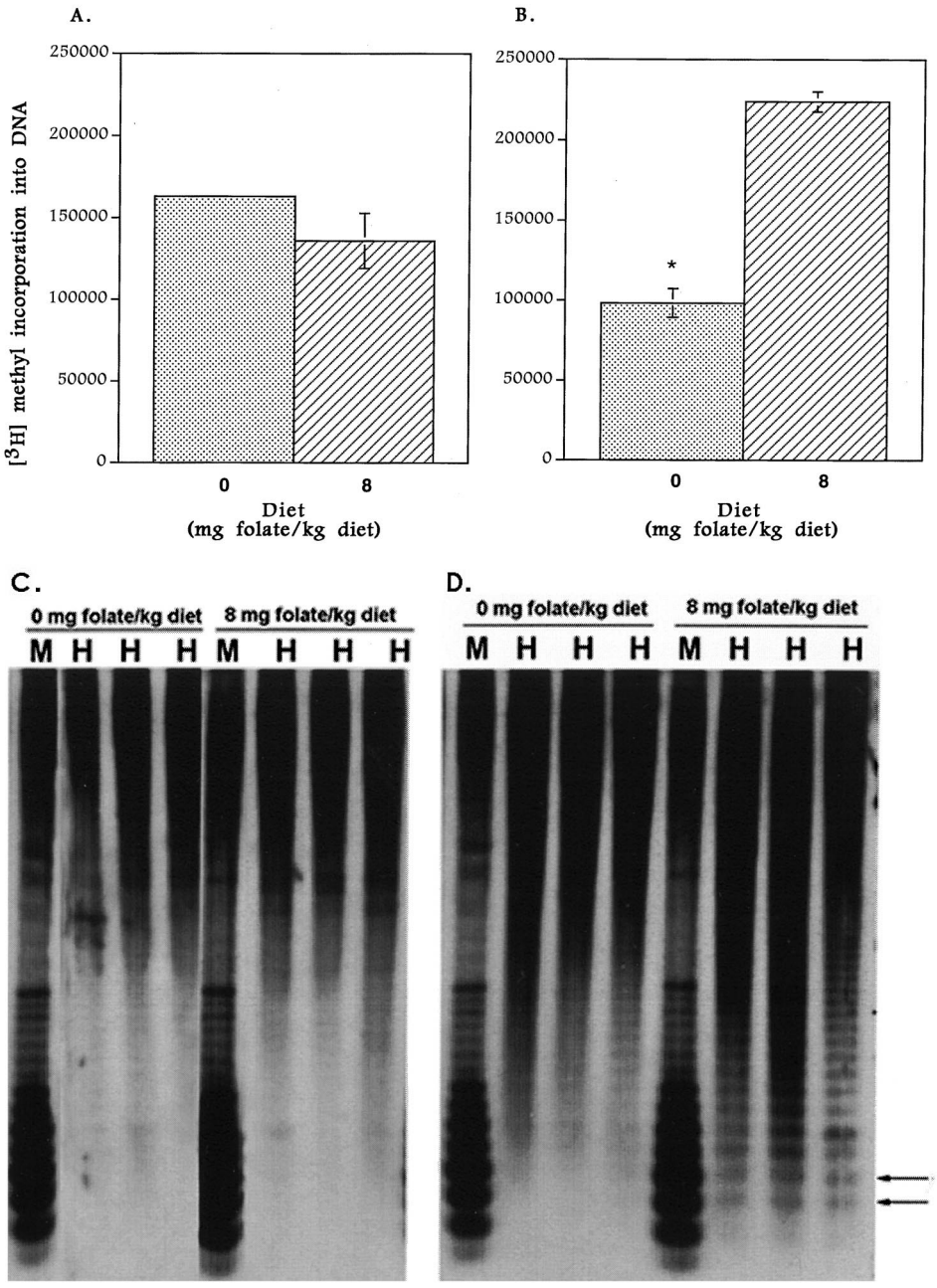

Fig. 2. The effect of dietary folate intervention on genomic DNA methylation. *A*, the *in vitro* methyl acceptance assay demonstrates no significant difference in *in vitro* [^{3}H]methyl incorporation (which is inversely related to the extent of *in vivo* genomic DNA methylation) into hepatic DNA between the folate-deficient and -sufficient animals at 8 weeks of intervention (*i.e.*, 3-week diet start). However, in *B*, the folate-deficient animals demonstrate a significantly lower degree of [^{3}H]methyl incorporation (*i.e.*, a higher degree of genomic DNA methylation) than the folate-supplemented animals at 5 weeks of intervention (*i.e.*, 6-week diet start; $*$, < 0.05). *C*, the methyl-sensitive restriction enzyme method also demonstrates no significant difference between the two groups when dietary folate intervention was provided for 8 weeks. However, in *D*, this assay demonstrates lower molecular weight *Hpa*II-digested fragments (*arrows*) in the folate-supplemented group compared with the folate-deficient group, which suggests a higher degree of genomic DNA methylation in the folate-deficient group than in the folate-supplemented group at 5 weeks of intervention. *H*, *Hpa*II- digested DNA samples; *M*: *Msp*I-digested DNA samples.

Effects of Dietary Folate Deficiency and Supplementation Beginning at 6 Weeks of Age (*i.e.*, after the Establishment of Neoplastic Foci) on Intestinal Tumorigenesis

Body Weight and Average Daily Food Consumption. The mean weight of the folate-supplemented mice was 16% greater than that of the folate-deficient mice at the start of the diet ($P < 0.03$, Fig. 1*B*). The mean weight of the folate-deficient mice increased in parallel to that of the folate-supplemented mice and remained 16–19% lower than that of the folate-supplemented mice at each time point throughout the experiment ($P < 0.03$, Fig. 1*B*). No premature deaths were observed. The average daily food consumption was also similar between the two groups (data not shown).

Serum Folate Concentrations. The mean serum folate concentration was significantly lower, by 4.8-fold, in mice placed on the folate-depleted diet than those on the supplemented diet ($P = 0.004$; Table 3). The mean serum folate concentrations of the folate-depleted and -supplemented mice were comparable with those observed in rats placed on corresponding diets (22, 23).

Development of Small Intestinal Adenomas and Colonic ACF and Adenomas. Mice fed the folate-deficient diet had a 4.2-fold lower number of small intestinal adenomas compared with those fed the folate-sufficient diet ($P = 0.001$; Table 3). When each of the segments of the small intestine was examined individually, this pattern was observed throughout the length of the small intestine ($P < 0.02$; Table 3).

In the colon, no significant difference was observed in the number of ACF and adenomas between the two diet groups (Table 3).

Genomic DNA Methylation Status. The mean incorporation of [^{3}H-methyl]SAM into hepatic DNA, which is inversely related to the extent of genomic DNA methylation, was 56% lower in the folate-deficient group than in the folate-supplemented group, which indicated a significantly higher degree of genomic DNA methylation in

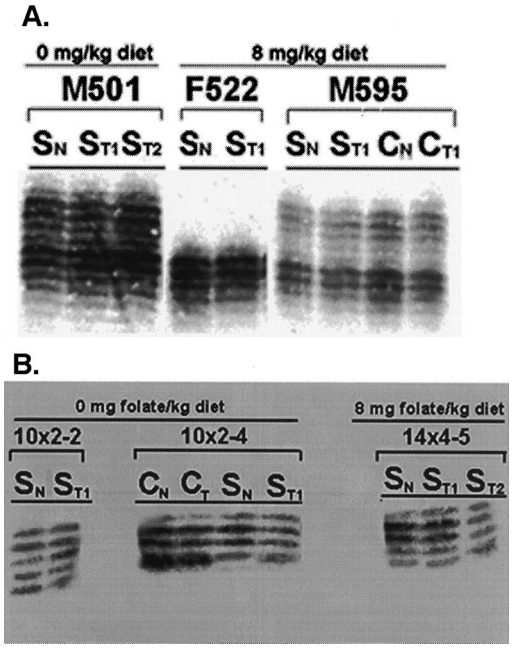

Fig. 3. Representative autoradiograms of microsatellite instability assay in *Apc*+/−*Msh2*−/− mice fed different levels of dietary folate. *A*, *D10 Mit2* locus assessed in mice fed diet before the establishment of neoplastic foci. *B*, *D5 Mit10* locus assessed in mice fed diet after the establishment of neoplastic foci. There was no difference in electrophoretic mobility of DNA from corresponding normal and small intestinal or colonic adenoma samples at either loci, indicating a lack of microsatellite instability. S_N: normal small bowel mucosal DNA; S_{Tn}: small bowel adenoma DNA (subscript n represents different samples); C_N, normal colon DNA; and C_{Tn}, colon adenoma DNA (subscript n represents different samples).

the folate-deficient group compared with the folate-supplemented group ($P < 0.05$; Fig. 2*B*). This finding was confirmed by the methyl-sensitive restriction digestion method, in which lower molecular weight *Hpa*II-digested fragments were observed in the folate-supplemented group compared with the folate-deficient group (Fig. 2*D*). These data suggest that a moderate degree of folate deficiency of 5 weeks duration paradoxically increased the extent of genomic DNA methylation.

Microsatellite Instability. A total of 20 small intestinal and colonic adenomas from 12 mice were analyzed for microsatellite instability. No difference in electrophoretic mobility was observed between normal and tumor tissue from either of the diet groups at the five loci tested (Fig. 3*B*).

DISCUSSION

The results of the present study suggest that dietary folate has strikingly different effects on intestinal tumorigenesis in *Apc*+/−*Msh2*−/− mice depending on the timing of intervention. Folate supplementation at four times the basal dietary requirement for rodents, started before the establishment of neoplastic foci, significantly decreased the number of small intestinal adenomas by 2.7-fold compared with a moderate degree of folate deficiency (Table 3). However, the number of small intestinal adenomas in the moderately folate-deficient mice was not significantly different from that observed in *Apc*+/−*Msh2*−/− mice on a control diet containing the basal dietary requirement of folate (*i.e.,* 2 mg folate/kg diet/day) for the same duration of time (38). This is likely related to the fact that by 11 weeks of age, the small intestine of *Apc*+/−*Msh2*−/− mice is maximally saturated with adenomas and, hence, no new adenomas can develop (38). Thus, the possibility that folate deficiency might have had an additional promoting effect on the development of small intestinal adenomas beyond the expected maximum number of adenomas observed in *Apc*+/−*Msh2*−/− mice on control diets, cannot be ruled out because the effect of this genotype is already maximal for any additional effects of folate deficiency to be seen.

In contrast to its effect given before the establishment of neoplastic foci, dietary folate had an opposite effect on the development of small intestinal adenomas when given after the establishment of neoplastic foci. In this situation, a moderate degree of folate deficiency induced by dietary depletion of folate significantly decreased the number of small intestinal adenomas by 4.2-fold compared with folate supplementation at four times the basal dietary requirement for rodents (Table 3). Dietary folate supplementation did not significantly increase the number of small intestinal adenomas beyond the maximum number of adenomas observed in *Apc*+/−*Msh2*−/− mice on a control diet containing the basal dietary requirement of folate for the same duration of time (38). Again, this is probably related to the saturation of the small intestine with adenomas by 11 weeks of age in *Apc*+/−*Msh2*−/− mice (38). Therefore, the possibility that folate supplementation might have had an additional promoting effect on the development of small intestinal adenomas beyond the expected maximum number of adenomas observed in *Apc*+/− *Msh2*−/− mice on control diets cannot be ruled out.

The effect of dietary folate on the development of colonic ACF and adenomas was different from that observed for small intestinal adenomas. Dietary folate supplementation, started before the establishment of neoplastic foci, significantly decreased the number of colonic ACF by 2.8-fold compared with a moderate degree of folate deficiency (Table 3). Although the folate-supplemented mice had a 67% reduction in the number of colonic adenomas compared with the folate-deficient mice, this fell short of statistical significance (Table 3). In contrast, dietary folate intervention started after the establishment of neoplastic foci had no significant effect on the development of colonic ACF and adenomas (Table 3).

The protective effect of dietary folate supplementation of a moderate degree (4 times the basal requirement) on small intestinal adenomas and colonic ACF, started before the establishment of neoplastic foci, in *Apc*+/− *Msh2*−/− mice corroborates previous studies in dimethylhydrazine-treated rats (22, 23). Our data also support prior epidemiological observations that suggest a 40% reduction in the risk of colorectal adenoma and cancer in individuals with the highest dietary folate intake compared with those with the lowest intake (3–16). The present study also confirms findings from a recent prospective study involving 88,756 female nurses, which showed a 75% reduction in colorectal cancer risk in women using multivitamin supplements containing $\geq$400 μg folic acid for $\geq$15 years after controlling for known confounding factors (17). Except for one study (8), however, it is not known whether dietary or supplemental folate taken in these epidemiological studies was started before neoplastic foci were present in the colon (3–17). Randomized folate intervention studies in human subjects free of colon adenomas or cancer are necessary to confirm our present findings in humans. Taken together, these observations suggest that a modest degree of folate supplementation may prevent the development of colorectal neoplasia. However, our study suggests that folate intervention should be started before the establishment of neoplastic foci.

Although the folate-supplemented mice had a 67% reduction in the number of colonic adenomas compared with the folate-deficient mice when intervention with dietary folate started before the establishment of neoplastic foci, this fell short of statistical significance (Table 3). This may be related to the fact that the number of histologically verified colonic adenomas per mouse was too few for an adequate statistical comparison. Another possibility is that the number of mice in each group was too small to detect a statistically significant differ-

ence in the number of colonic adenomas between these two groups (*i.e.,* type II error). This finding concerning colonic adenomas may seem to be inconsistent compared with the protective effect observed for colonic ACF. However, although *Apc+/− Msh2−/−* mice develop numerous ACF, these ACF do not seem to contribute significantly to the colonic adenoma population (38). What factors limit the progression of ACF to adenomas in this model is unclear. It may simply be a lack of time; there may not be enough time for adenomas to develop in these mice inasmuch as they have a life span of only 11–13 weeks (38). It also seems that ACF develop postnatally whereas colonic adenomas arise perinatally in these mice (38).

The inhibitory effect of dietary folate deficiency started after the establishment of neoplastic foci, on small intestinal tumorigenesis in the present study, is not entirely surprising. Folate is an important factor for a number of metabolic pathways that involve the transfer of one-carbon groups (58). Among such pathways are the biosyntheses of purines and thymidylates, and as such, folate plays a key role in DNA replication and cell division (58). Consequently, folate deficiency in tissues with rapidly replicating cells results in ineffective DNA synthesis. In neoplastic cells, in which DNA replication and cell division are occurring at an accelerated rate, interruption of folate metabolism has been observed to cause ineffective DNA synthesis, which results in the inhibition of tumor growth (59). Indeed, this has been the basis for antitumor therapy using a number of antifolate agents, including methotrexate and 5-fluorouracil (59). It has been shown in experimental models that growth of a transplanted cancer is inhibited in folate-deficient rats (60), that folate deprivation reduces the growth of virally induced cancers (61), and that the time required for developing nerve sheath tumors in transgenic mice is significantly delayed by restricting the level of folate in diet (62). Furthermore, the addition of folate to established tumors has been shown to result in an "acceleration phenomenon." For instance, children with acute leukemia treated with folate supplementation experienced an accelerated progression of leukemia (63). Taken together, these observations suggest that folate deficiency, started after the establishment of neoplastic foci, has an inhibitory effect of tumor progression or may even cause tumor regression.

In contrast to its inhibitory effect on small intestinal adenomas, dietary folate deficiency, started after the establishment of neoplastic foci, had no significant effect on the number of ACF and adenomas in the colon. It may be that rapidly developing small intestinal adenomas with accelerated tumorigenesis in these mice are most susceptible to the effect of folate depletion, whereas colonic ACF and adenomas with a normal or slower growth rate may not. Another possibility is a lack of time for folate deficiency to exert its effect on established colonic neoplastic foci because most mice die by 11 weeks of age (38).

The mechanisms by which dietary folate can modulate intestinal tumorigenesis have not been clearly elucidated. In the present study, two of the proposed mechanisms, genomic DNA methylation and mismatch repair, were investigated. Folate is an important factor in DNA methylation (64), which is an important epigenetic determinant in gene expression (an inverse relationship), in the maintenance of DNA integrity and stability, and in the development of mutations (65, 66). Genomic and proto-oncogene-specific DNA hypomethylation seems to be an early, and consistent, event in carcinogenesis (65, 66) including that of colorectal cancer (30, 53, 67). In addition, site-specific hypermethylation at specific CpG islands located near or at the promoter region of tumor suppressor and mismatch repair genes seems to be an important mechanism in gene silencing in colorectal carcinogenesis (68, 69).

In the present study, a moderately folate-deficient diet, provided for 8 weeks (*i.e.,* a 3-week start), did not produce a significant degree of genomic DNA hypomethylation in the liver compared with a folate-supplemented diet (Fig. 2, *A* and *C*). This finding is consistent with previous observations made in rats subjected to the similar diets (23, 70). This suggests that alterations in genomic DNA methylation are not a major mechanism by which folate supplementation that started before the establishment of neoplastic foci significantly reduced the number of small intestinal adenomas and colonic ACF in *Apc+/− Msh2−/−* mice. In contrast, a moderately folate-deficient diet, provided for 5 weeks (*i.e.,* a 6-week start), increased the extent of genomic DNA methylation in the liver compared with a folate-supplemented diet in the present study (Fig. 2, *B* and *D*). This finding may appear paradoxical but is consistent with a prior observation that demonstrated that severely folate-depleted rats had a 59% increase in the extent of genomic DNA methylation in the liver compared with control rats at 6 weeks of folate depletion (71). This may be related to the observations that states associated with diminished availability of SAM result in an enhancement of DNA methyltransferase activity, the enzyme responsible for DNA methylation (72, 73). Therefore, moderate folate deficiency of 5 weeks is associated with an increased degree of genomic DNA methylation attributable to a compensatory up-regulation of DNA methyltransferase, but, by 8 weeks of folate deficiency, this effect is no longer observed. The increased extent of genomic DNA methylation associated with moderate folate-deficiency of 5 weeks' duration was associated with a significant reduction in the number of small intestinal adenomas compared with folate-supplementation.

Because the small intestines and colons of the *Apc+/− Msh2−/−* mice were covered with numerous adenomas and ACF, it was technically impossible to scrape only the normal mucosa excluding adenomas and ACF to obtain normal small intestinal and colonic DNA. Determining the extent of genomic DNA methylation from intestinal mucosal scrapings containing varying proportions of normal cells, adenomas, and ACF would not have been informative. In the present study, therefore, the effect of dietary folate on genomic DNA methylation in normal cells was determined only in the liver. Because both site-specific hypo- and hypermethylation play a role in carcinogenesis (65, 66) and because folate may modulate DNA methylation in a site-specific manner (71), it would be of interest to study the effect of folate on sites within proto-oncogenes and tumor suppressor and mismatch repair genes that are implicated in intestinal carcinogenesis from microdissected normal cells, adenomas, and ACF.

Another mechanism that was investigated in the present study pertains to the effect of folate on DNA mismatch repair. Folate is an essential factor for the *de novo* biosynthesis of purines and thymidylate and, hence, plays an important role in the maintenance of DNA integrity and stability (58). Accumulating *in vivo* and *in vitro* evidence suggests that folate deficiency is associated with DNA damage (71, 74), deoxynucleotide pool imbalances leading to misincorporation of uracil into newly synthesized DNA (74, 75), and impaired DNA excision (76) and mismatch (77) repair.

The present study, however, suggests that widespread microsatellite instability, which is observed in the majority of HNPCC and 15–20% of sporadic colorectal cancers in humans (43), is likely not a major factor leading to accelerated small intestinal and colonic tumorigenesis in *Apc+/− Msh2−/−* mice. Even folate deficiency and supplementation had no significant effect on microsatellite instability, which suggests that modulation of microsatellite instability is not likely a mechanism by which dietary folate modulates small intestinal and colonic tumorigenesis in this model. The microsatellite instability phenotype is observed in some mismatch repair-deficient murine models including *Msh2−/−* (44, 45), *Mlh1−/−* (78), and *Pms2−/−* (79) mice, whereas it is not seen in other models such as *Msh6−/−* (80). Although lymphoid and skin cancers and a small fraction of

small intestinal and colonic cancers from *Msh2*−/− mice (44, 45) display microsatellite instability, it is rarely observed in small intestinal and colonic polyps from *Apc*+/− *Msh2*−/− mice (38). Despite the lack of widespread microsatellite instability in *Apc*+/− *Msh2*−/− mice, it is possible that *Msh2* deficiency may cause replication errors in one or a few critical genes resulting in accelerated intestinal tumorigenesis.

Several limitations associated with the *Apc*+/− *Msh2*−/− murine model need to be acknowledged: (*a*) the predominant phenotype in this model is the development of small intestinal adenomas in contrast to colon polyps in humans; (*b*) *Apc*+/− *Msh2*−/− mice do not develop small intestinal or colonic adenocarcinoma because they become moribund as a consequence of florid polyposis; (*c*) as previously described, the contribution of ACF to the adenoma population in this model is not clearly established; (*d*) this model may reflect only inherited types of accelerated tumorigenesis such as familial adenomatous polyposis and HNPCC and not sporadic colorectal carcinogenesis. Lastly, despite *Msh2* deficiency, widespread microsatellite instability is absent in tumors. Nonetheless, the *Apc*+/− *Msh2*−/− mouse seem to be an excellent model to study chemopreventive effects of dietary factors and drugs on colorectal carcinogenesis because of the spontaneous development of small intestinal and colonic adenomas and ACF, genetic similarities to human colorectal cancer, and the accelerated nature of tumorigenesis, which provides an opportunity to determine effects of chemopreventive agents on both initiation and progression of tumorigenesis in a relatively short time.

The mice that were randomized to receive 0 mg folate/kg for the 6-week start study had an unexpected 16% lower mean weight at the start of the diet and remained 16–19% lower than the folate-supplemented mice during the 5 weeks of dietary intervention despite similar average daily food consumption (Fig. 1*B*). However, the folate-deficient mice demonstrated no growth retardation and their mean weight increased in parallel to that of the folate-supplemented mice during the study period (Fig. 1*B*). Because calorie restriction may inhibit tumor development in chemical and genetic knockout models (81, 82), the possibility that the significantly lower weights of the folate-deficient mice might have contributed to lower numbers of small intestinal adenomas compared with the folate-supplemented diet when folate intervention was started at 6 weeks of age (i.*e.*, after the establishment of neoplastic foci) cannot be ruled out. However, one observation against this possibility is that the folate-deficient mice had significantly higher numbers of small intestinal adenomas and colonic ACF compared with the folate-supplemented mice when dietary intervention was started at 3 weeks of age (*i.e.*, before the establishment of neoplastic foci) despite the fact that their mean weight was 8–18% lower (albeit statically nonsignificant) compared with the folate-supplemented mice during the study period (Fig. 1*A*). The present study did not include a group receiving a diet containing the basal dietary requirement of folate (*i.e.*, 2 mg folate/kg diet) and comparisons were made to a historic control receiving this diet from a previous study (38). Future studies examining the dose-response effect of dietary folate in this model are warranted to confirm our present findings.

In summary, the present study suggests that dietary folate supplementation, at 4 times the basal requirement, significantly protects against the development of small intestinal adenomas and colon ACF if started before the establishment of neoplastic foci, in this genetically predisposed mouse intestinal tumorigenesis model. However, if started after the establishment of neoplastic foci, dietary folate seems to have an opposite effect in this model. These data suggest that the timing of folate intervention is critical in providing an effective and safe chemopreventive effect on colorectal carcinogenesis. Notwithstanding the limitations associated with this model, our data suggest that the optimal timing of folate intervention should be established before folate supplementation can be used as a safe chemopreventive agent against colorectal cancer. Furthermore, the mechanisms by which folate modulates colorectal carcinogenesis need to be elucidated.

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