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Genetic and nutritional deficiencies in folate metabolism influence tumorigenicity in $Apc^{min/+}$ mice

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Abstract

Epidemiological studies indicate that adequate dietary folate is protective against colon cancer, although mechanisms remain largely elusive. We investigated the effects of genetic disruptions of folate transport and metabolism and of dietary folate deficiency in a mouse model of colon cancer, the $Apc^{min/+}$ mouse. $Apc^{min/+}$ mice with heterozygous knockout of the gene for reduced folate carrier 1 ($Rfc1^{+/-}$) developed significantly fewer adenomas compared to $Rfc1^{+/+}Apc^{min/+}$ mice [30.3±4.6 vs. 60.4±9.4 on a control diet (CD) and 42.6±4.4 vs. 55.8±7.6 on a folate-deficient diet, respectively]. $Rfc1^{+/-}Apc^{min/+}$ mice also carried a lower tumor load, an indicator of tumor size as well as of tumor number. In contrast, there were no differences in adenoma formation between $Apc^{min/+}$ mice carrying a knockout allele for methionine synthase ($Mtr^{+/-}$), an enzyme that catalyzes folate-dependent homocysteine remethylation, and $Mtr^{+/+}Apc^{min/+}$ mice. However, in both *Mtr* groups of mice, dietary folate deficiency significantly increased adenoma number (from 32.3±3.8 on a CD to 48.1±4.2 on a folate-deficient diet), increased plasma homocysteine, decreased global DNA methylation in preneoplastic intestines and increased apoptosis in tissues. There were no genotype-associated differences in these parameters in the *Rfc1* group, suggesting that the protection conferred by *Rfc1* deficiency is carried out through a different mechanism. In conclusion, genetic and nutritional disturbances in folate metabolism can have distinct influences on tumorigenesis in $Apc^{min/+}$ mice; altered levels of homocysteine, global DNA methylation and apoptosis may contribute mechanistically to dietary influence.
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Keywords: Folate metabolism; Folate transport; Tumorigenesis; Intestinal neoplasia

1. Introduction

Folate derivatives participate in several important cellular processes, including amino acid interconversions and DNA methylation, synthesis and repair. Epidemiological studies have demonstrated that dietary folate status and the activity of folate-dependent enzymes can alter the risk of certain cancers. Specifically, several studies have shown that high folate intake is inversely correlated with the risk of developing colorectal adenomas and carcinomas and that an activity-reducing polymorphism in an enzyme central to folate metabolism [methylenetetrahydrofolate reductase (MTHFR)] has a protective effect against colon cancer incidence when adequate folate status is maintained [1–4]. The mechanisms by which folate modulates cancer risk are not completely understood, although several hypotheses have been proposed.

Folate is required for the generation of *S*-adenosylmethionine (SAM), an important methyl donor involved in the DNA methyltransferase (Dnmt)-catalyzed methylation of DNA, a means of transcriptional regulation. Folate defi-

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ciency may cause global DNA hypomethylation (potentially permitting oncogene expression) or aberrant methylation patterns, such as promoter hypermethylation (preventing the expression of tumor-suppressor genes). For example, a severely methyl-deficient diet was shown to induce DNA hypomethylation and to increase mRNA levels of the *c-myc* and *c-fos* proto-oncogenes in the rat liver [5]. In contrast, another study using a rat colon cancer model did not observe any changes in DNA methylation during folate deficiency, although the number and size of colonic aberrant crypt foci (ACF) were reduced [6].

The de novo synthesis of nucleotides is also dependent on the availability of folate. 10-FormylTHF contributes one-carbon units for the generation of purines, and 5, 10-methyleneTHF provides the methyl group for the methylation of dUMP to dTMP. In vitro studies suggest that the latter reaction is critical since a high dUTP/dTTP ratio may lead to the misincorporation of uracil into DNA and ultimately to DNA double-strand breaks, genomic instability and DNA fragmentation [7–12]. Folate deficiency therefore leads to uracil misincorporation and DNA damage, as well as to a reduced pool of nucleotides for DNA synthesis and repair.

Disturbances of folate metabolism can also adversely affect the rate of apoptosis in animal tissues [10,13]. This may be a result of methylation or nucleotide pool changes (as discussed above), an accumulation of cytotoxic homocysteine or a deficiency of choline (the precursor of betaine), an alternate carbon donor for homocysteine remethylation to methionine. Enhanced apoptosis can be beneficial in eliminating tumorigenic cells, thus inhibiting the formation — or restricting the size — of tumors. However, it may also induce a chronic increase in cell turnover rate, decreased repair time and increased DNA damage, with a selection pressure that favors cells with transformation potential [14].

Mouse models provide an opportunity to explore the etiology of complex diseases. An established model for intestinal neoplasia, the *Apc*$^{min/+}$ mouse, develops multiple small intestinal adenomas within a few months of age [15]. These mice harbor a germline mutation in the tumor suppressor *Apc*, a gene shown to be frequently mutated in sporadic and hereditary forms of colon cancer in humans [16]. The effect of dietary folate has been investigated in these mice, with variable outcomes that might be dependent on the timing and duration of intervention. In one study, there was no difference in total adenoma number between folate-supplemented and folate-deficient mice, although there was a decrease in the number of ileal adenomas in the folate-supplemented group at 3 months and a decrease in ileal adenomas in the folate-deficient group at 6 months [17]. In our previous work with *Apc*$^{min/+}$ mice, we concluded that folate deficiency exerts different effects depending upon the transformation state of the cell. When folate intervention occurs at an early stage, it may promote tumorigenesis; if it occurs during later stages of transformation, it may inhibit tumor growth [18].

Apc$^{min/+}$ mice have also been used to investigate genetic modifiers of cancer. In multiple reports, Dnmt deficiency significantly reduced adenoma multiplicity [19–21], whereas *Mlh*- or *Msh*-null mutations increased adenoma number dramatically [22,23]. In one study, a moderately folate-deficient diet in *Apc*$^{min/+}$ mice carrying a *Dnmt1* mutation did not alter global DNA methylation or promoter-specific methylation of the *E-cadherin* gene [21]. In another report, Dnmt deficiency was associated with a decrease in the methylation of CpG islands of *Itga4*, *Mgmt* and *Timp3*, with a decrease in tumor number [20].

Despite the critical role of folate metabolism in tumorigenesis, crosses between mouse models of folate transport and metabolism and *Apc*$^{min/+}$ mice have not yet been reported. We therefore examined the influence of genetic and nutritional disturbances in folate metabolism on tumorigenicity in *Apc*$^{min/+}$ mice by crossing them with mice harboring a knockout allele in one of two key genes in folate transport and metabolism and by administering control and folate-deficient diets to these animals. The mouse models were heterozygous for the disruption of the gene for reduced folate carrier 1 (Rfc1) or for the gene encoding methionine synthase (MTR). Rfc1 transports the predominant plasma folate, 5-methylTHF, into cells. MTR transfers the methyl group from 5-methylTHF to homocysteine to generate methionine, which is subsequently converted to SAM. We investigated the impact of these metabolic disruptions on tumor multiplicity and size in *Apc*$^{min/+}$ mice, in addition to cellular processes that might contribute to tumorigenesis, specifically global DNA methylation and intestinal apoptosis.

2. Materials and methods

2.1. Mice and dietary intervention

Animal experimentation was approved by the Montreal Children's Hospital Animal Care Committee, in accordance with the guidelines of the Canadian Council on Animal Care.

The generation and genotyping of *Rfc1*$^{+/-}$ and *Mtr*$^{+/-}$ were performed as described previously [24,25]. *Rfc1* mice were established on SWV/Fnn background [24]. *Mtr* mice were established on a mixed Black Swiss and 129SV background [25]. Male *Apc*$^{min/+}$ mice (C57BL/6J background) were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred with female *Rfc1*$^{+/-}$ or *Mtr*$^{+/-}$ mice. Offspring were weaned at 3–3.5 weeks and fed amino-acid-defined diets (Harlan Teklad, Madison WI) until they were 10 weeks old. These diets complied with the recommendations of the American Institute of Nutrition guidelines for rodents [26]. The mice were randomly assigned to receive either a control diet (CD) containing 2 mg folic acid/kg diet (recommended amount) or a folic-acid-deficient diet (FADD) containing 0.2–0.3 mg folic acid/kg diet. All diets were supplemented with 1% succinylsulfanthiozole to inhibit folic acid synthesis by intestinal flora.

All mice were genotyped for *Pla2g2a*, the candidate gene for *Mom1*, an established modifier of the Min phenotype that can affect adenoma formation, using described procedures [27]. This was performed to ensure that the four experimental groups for each mouse strain were of the same *Pla2g2a* genotype. There are two possible alleles: $Mom1^R$ (wild-type *Pla2g2a*), which confers $Apc^{min/+}$ mice resistance to adenoma formation, and $Mom1^S$ (mutant *Pla2g2a*), which sensitizes to adenoma formation. Every mouse in the *Rfc1* group was $Mom1^{S/R}$ (the $Apc^{min/+}$ strain is $Mom1^{S/S}$ and the *Rfc1* strain is $Mom1^{R/R}$, as genotyped in our laboratory). *Mtr* mice were either $Mom1^{S/S}$ or $Mom1^{S/R}$ due to their mixed background (genotyping of the parental strains in our laboratory revealed that the Black Swiss strain is $Mom1^R$ and the 129SV is $Mom1^S$). Since $Apc^{min/+}$ mice are $Mom1^{S/S}$, we included *Mtr* mice that were $Mom1^{S/S}$ for our experiments. For this reason, one group ($Mtr^{+/-}$ FADD) was left with only three mice, and further attempts at breeding mice of the same *Mom1* genotype were not successful.

2.2. Adenoma scoring

The number and size of small intestinal adenoma were determined as in our previous reports [18,21]. Mice were sacrificed by asphyxiation. Blood was obtained by cardiac puncture for plasma homocysteine evaluation. Intestines were removed, opened longitudinally and flushed with phosphate-buffered saline. Selected adenomas and preneoplastic (normal) intestinal tissues were snap-frozen in liquid nitrogen and stored at −70°C. The remaining intestine was placed flat between two pieces of filter paper and fixed in 10% formalin solution for at least 24 h. The intestine was then stained with a 0.1% methylene blue solution and assessed for adenoma number and size by two different individuals blinded to genotype and diet using a dissecting microscope and a micrometer. Adenoma load refers to the sum of the areas of tumors for each mouse.

2.3. Total plasma homocysteine (tHcy) evaluation

Cardiac blood was collected in potassium–EDTA tubes and centrifuged at 6000×*g* for 5 min at 4°C to separate plasma. Measurements were performed by high-performance liquid chromatography, as described [28].

2.4. Methylation analysis

To assess the methylation of CCGG sites in preneoplastic (normal) intestines, a thin-layer chromatography (TLC) assay was performed as previously described [29]. Briefly, 5 μg of RNA-free genomic DNA was treated with *Msp1* (which digests both methylated and unmethylated CCGG sequences). The DNA was then treated with calf intestinal alkaline phosphatase, end-labeled with [^{32}P]γdATP, hydrolyzed with nuclease P1, spotted on a cellulose TLC plate and developed in isobutyric-acid–water–ammonium hydroxide (66:33:1). The images were quantified by a phosphorimager. The amount of methylation was calculated as the percentage of methylated cytosines/(methylated cytosines+unmethylated cytosines).

2.5. Caspase-3/7 activity assay

Preneoplastic intestinal tissue was ground into powder in liquid nitrogen and lysed in buffer (50 mmol/L potassium phosphate, 0.3 mmol/L EDTA, pH 8.0). Total protein concentration was determined using Bio-Rad Protein Assay solution (Bio-Rad, Montreal, Canada). Caspase-Glo 3/7 Assay kit (Promega, Nepean, Canada) was used to measure caspase-3/7 activities, according to the manufacturer's instructions. Two micrograms of total protein was used per assay. Two different intestinal extracts from the same mouse, each assayed in duplicate, were used to generate a mean of four assays per mouse.

2.6. Statistical analysis

Two-factor analysis of variance (ANOVA) and independent-sample *t* tests were performed using SPSS for WINDOWS software, version 11.0. $P<.05$ was considered significant. All data are reported as mean±S.E.M.

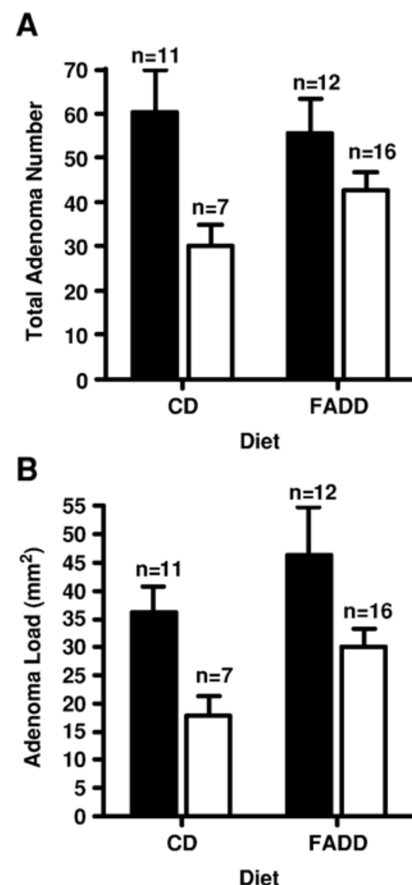

Fig. 1. Effect of *Rfc1* genotype and dietary folate on (A) adenoma number and (B) adenoma load in $Rfc^{+/+}Apc^{min/+}$ (■) and $Rfc^{+/-}Apc^{min/+}$ (□) mice on CD or on FADD. Values represent mean±S.E.M. n=number of mice.

3. Results

3.1. Adenoma number and load in $Apc^{min/+}$ mice are reduced by Rfc1 deficiency

$Rfc1^{+/-}Apc^{min/+}$ mice developed fewer adenomas than did $Rfc1^{+/+}Apc^{min/+}$ mice (Fig. 1A; $P<.05$, two-factor ANOVA). On the control folate-repleted diet, $Rfc1^{+/-}Apc^{min/+}$ mice had 50% fewer tumors than their wild-type $Rfc1^{+/+}Apc^{min/+}$ counterparts (30.3±4.6 vs. 60.4±9.4). The values for the folate-deficient diet were 42.6±4.4 versus 55.8±7.6. The total area of the adenomas (expressed as adenoma load) was also lower in $Rfc1^{+/-}Apc^{min/+}$ mice than in $Rfc1^{+/+}Apc^{min/+}$ mice (Fig. 1B; $P<.05$, two-factor ANOVA). The load on CD for $Rfc1^{+/-}Apc^{min/+}$ and $Rfc1^{+/+}Apc^{min/+}$ was 17.9±3.3 and 36.1±4.7 mm^2; the load on the folate-deficient diet was 29.0±3.3 and 46.5±8.5 mm^2, respectively.

Adenoma load was slightly but nonsignificantly increased by FADD (Fig. 1B; $P=.07$, two-factor ANOVA). Since the average adenoma size was also slightly but not significantly increased (data not shown), it is likely that the increase in load is attributable to an increase in adenoma size.

To examine the potential effects of gender, we repeated each ANOVA with gender as a covariate; both number and load remained significant for genotype ($Rfc1^{+/+}$, 10 females, 13 males; $Rfc1^{+/-}$, 12 females, 11 males).

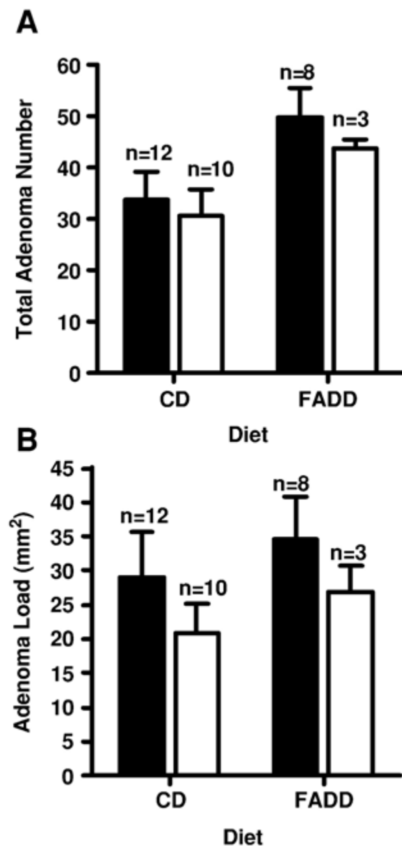

Fig. 2. Effect of *Mtr* genotype and dietary folate on (A) adenoma number and (B) adenoma load in $Mtr^{+/+}Apc^{min/+}$ (■) and $Mtr^{+/-}Apc^{min/+}$ (□) mice on CD or on FADD. Values represent mean±S.E.M. n = number of mice.

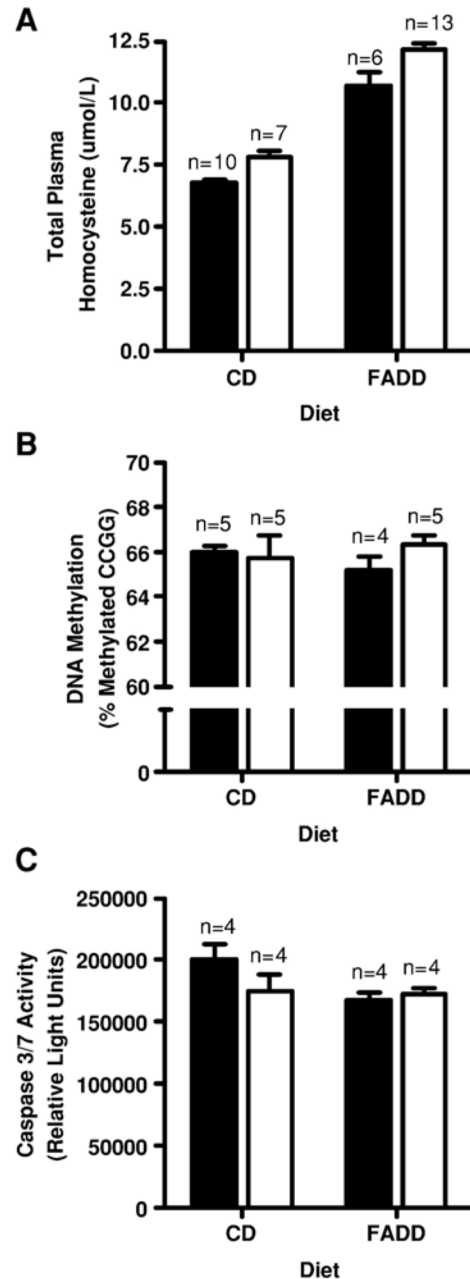

Fig. 3. Effect of *Rfc1* genotype and dietary folate on (A) tHcy, (B) intestinal global DNA methylation and (C) intestinal caspase-3/7 activity in $Rfc^{+/+}Apc^{min/+}$ (■) and $Rfc^{+/-}Apc^{min/+}$ (□) mice. Values represent mean±S.E.M. n = number of mice.

3.2. Dietary folate deficiency increases adenoma number in $Mtr^{+/+}Apc^{min/+}$ and $Mtr^{+/-}Apc^{min/+}$ mice

Mtr genotype did not significantly affect either adenoma number or load (Fig. 2A and B). However, adenoma number was increased in both genotype groups ($Mtr^{+/+}Apc^{min/+}$ and $Mtr^{+/-}Apc^{min/+}$ mice) receiving FADD (Fig. 2A; $P<.05$, two-factor ANOVA), from an average of 32.3±3.8 on CD to 48.1±4.2 on FADD. There was no significant dietary influence on adenoma load (Fig. 2B). When analyses were repeated with gender as a covariate, the significance of diet

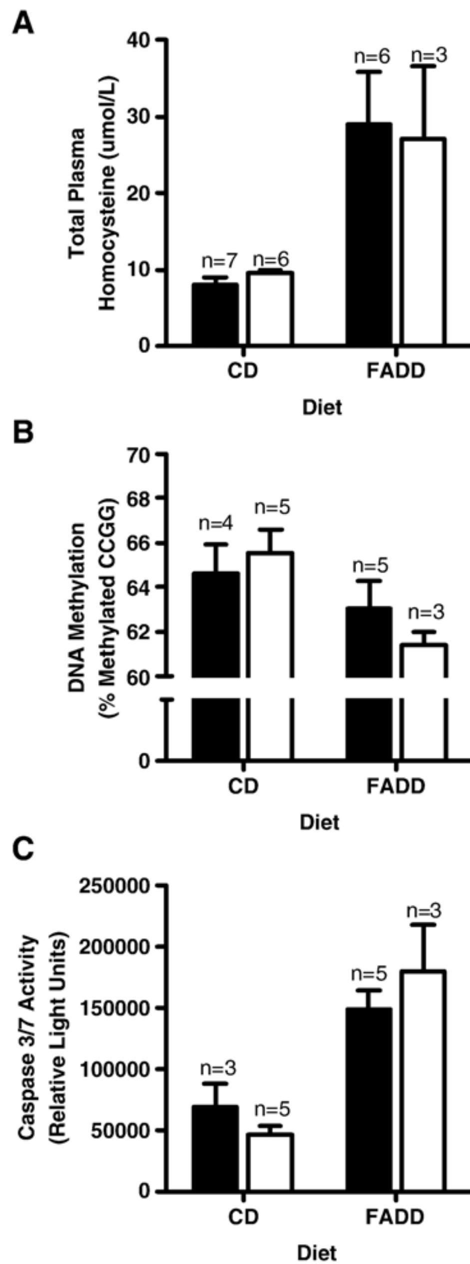

Fig. 4. Effect of *Mtr* genotype and dietary folate on (A) tHcy, (B) intestinal global DNA methylation and (C) intestinal caspase-3/7 activity in $Mtr^{+/+}Apc^{min/+}$ (■) and $Mtr^{+/-}Apc^{min/+}$ (□) mice. Values represent mean±S.E.M. *n*=number of mice.

effect on adenoma number was retained (CD, 6 females, 16 males; FADD, 6 females, 5 males).

3.3. Dietary folate, but not Rfc1 or Mtr genotype, affects tHcy, global DNA methylation and levels of apoptosis in $Mtr^{+/+}Apc^{min/+}$ and $Mtr^{+/-}Apc^{min/+}$ mice

Homocysteine is a cytotoxic amino acid that is either eliminated via transsulfuration or remethylated to form methionine, using either 5-methylTHF or betaine as a methyl donor. Since mice have been shown to develop mild hyperhomocysteinemia in response to FADDs [30], we measured tHcy to ensure the effectiveness of FADD and to determine if hyperhomocysteinemia or its consequences could contribute to observed decreases in adenoma number or load. There was no influence of genotype on plasma homocysteine in the two strains on either diet. In contrast, the folate-deficient diet significantly increased homocysteine in $Rfc1^{+/+}Apc^{min/+}$ and $Rfc1^{+/-}Apc^{min/+}$ mice, as well as in $Mtr^{+/+}Apc^{min/+}$ and $Mtr^{+/-}Apc^{min/+}$ mice, but the increase was much more dramatic in the *Mtr* group of $Apc^{min/+}$ mice, which demonstrated a threefold increase in plasma homocysteine on FADD compared to the value on CD; the increase due to diet in the *Rfc1* group of $Apc^{min/+}$ mice was approximately 50% (Figs. 3A and 4A).

SAM is the methyl donor for Dnmt-catalyzed DNA methylation, an important process in epigenetic gene regulation. Increased levels of homocysteine will generate SAH, an inhibitor of SAM-dependent methyltransferases. In the *Mtr* group, which demonstrated a dramatic diet-dependent increase in homocysteine, there was a concomitant decrease in DNA methylation in the preneoplastic intestinal tissue (Fig. 4B). *Rfc1* mice, which did not respond as dramatically to FADD in terms of plasma tHcy, did not exhibit any significant changes in global DNA methylation in the intestine (Fig. 3B). There were no significant effects of *Mtr* or *Rfc1* genotype on methylation in the preneoplastic intestinal DNA of these $Apc^{min/+}$ mice; this observation is consistent with the absence of an effect of genotype on homocysteine levels.

Since homocysteine has been reported to increase apoptosis in some experimental systems, we also assessed caspase-3/7 activities as a marker of apoptosis in preneoplastic intestinal tissues. In $Mtr^{+/+}Apc^{min/+}$ and $Mtr^{+/-}Apc^{min/+}$ mice, caspase-3/7 activities were increased on FADD (Fig. 4C), indicating an increased rate of apoptosis in this tissue. $Rfc1^{+/+}Apc^{min/+}$ and $Rfc1^{+/-}Apc^{min/+}$ mice did not exhibit any significant effects of diet on caspase activities (Fig. 3C). As mentioned for methylation, there was no effect of genotype on apoptosis; this is also consistent with the absence of a genotype effect on plasma homocysteine.

Basal levels of intestinal apoptosis on CD were different between *Rfc1* $Apc^{min/+}$ mice and *Mtr* $Apc^{min/+}$ mice, but this could be attributable to strain differences; we tested additional strains and obtained variable strain-dependent levels of apoptosis (data not shown).

4. Discussion

This study demonstrates that both genetic and nutritional disturbances in folate metabolism can influence adenoma formation and growth in $Apc^{min/+}$ mice. Genetic disruption of the *Rfc1* gene reduced adenoma number and load in this strain, whereas low dietary folate did not have a significant impact. In contrast, dietary folate deficiency increased tumor number in $Mtr^{+/+}Apc^{min/+}$ and $Mtr^{+/-}Apc^{min/+}$ mice, whereas genetic mutation had little or no influence. Direct

comparisons between the two mutant strains cannot be made since they are on different genetic backgrounds. Nonetheless, our work suggests that folate metabolism can influence tumorigenesis in a multifactorial manner, depending on the genetic variation and nutritional status of the host. This was underscored further by the fact that $Rfc^{+/+}Apc^{min/+}$ mice, all of which carry a $Mom1^{R}$ allele, developed more adenomas overall than the $Mtr^{+/+}Apc^{min/+}$ group, which did not. The genetic background of *Rfc* mice may contain additional modifiers of the Min phenotype that can increase adenoma number.

Rfc1 mice are on a uniform genetic background (SWV/Fnn) [24], whereas *Mtr* mice are on a mixed background (Black Swiss and 129SV) [25]. Since strong genetic determinants of tumorigenesis may be less evident on a mixed background, this factor may have contributed to the dramatic effect of diet in $Mtr^{+/+}Apc^{min/+}$ and $Mtr^{+/-}Apc^{min/+}$ mice.

The genetic deficiency of *Rfc1*, a reduced folate transporter, resulted in fewer adenomas with no concurrent changes in plasma homocysteine, global DNA methylation or intestinal cell apoptosis. Low dietary folate, administered to rodents postnatally, has been examined for its role in tumor formation in the $Apc^{min/+}$ mouse model, as well as in other colon cancer models [18,21,31]. Here, we show that a very early disruption, in the form of a genetic defect in folate transport, inhibits adenoma formation in these mice, although the mechanism remains speculative.

Plasma homocysteine in the *Rfc1* strain was not influenced by *Rfc1* mutation and was only modestly affected by low dietary folate (~50% increase on FADD). These findings, which may relate to the background of the strain, are consistent with those of a recent study, which showed that these mice were refractory to folate deficiency with respect to changes in the levels of SAM and SAH — other biomarkers in the homocysteine/methylation cycle [32].

In adult mice, the intestinal expression of *Rfc1* in $Rfc1^{+/-}$ mice, based on reverse transcription polymerase chain reaction, is lower than that of their wild-type littermates (data not shown), and there are modest differences in colonic mucosal SAM and SAH metabolites [24]. Developmentally, however, *Rfc1* expression may be more critical; homozygous mutants are embryonic lethal [32], and nursing mice receive 5-methyTHF from their mother's breast milk [33]. This is noteworthy for studies involving $Apc^{min/+}$ mice, since their intestinal cells have an age-specific sensitivity to adenoma formation. Younger (5- to 15-day-old) mice are more sensitive to adenoma initiation by chemical carcinogen exposure than older mice [34]. Throughout development and in the first few postnatal weeks, mice are dependent on maternal sources of folate and on their own uptake mechanisms. Consequently, a genetic defect in *Rfc1* transporter might manifest itself earlier than a dietary deficiency that is initiated on weaning. The modulation of adenoma initiation rates could occur through several potential mechanisms. For example, polyamines are necessary for cell proliferation and can be formed from the decarboxylation of SAM. *Rfc1* heterozygosity has previously been shown to decrease the SAM/SAH ratio [24]. By genetically depleting polyamines through increased catabolism, it was shown that $Apc^{min/+}$ mice develop 75% fewer adenomas than do wild-type mice [35]. Although we did not observe changes in global DNA methylation in this strain, *Rfc1* heterozygosity could potentially result in methylation changes within critical oncogenes or tumor-suppressor genes. In addition, the pool of DNA precursors for proliferation may have been reduced, inhibiting the DNA synthesis of burgeoning adenomas.

A decrease in adenoma number was also found in folate-deficient $Rfc1^{+/-}Apc^{min/+}$ mice, compared to $Rfc^{+/+}Apc^{min/+}$ mice, although this was not statistically significant when measured by *t* test (55.8±7.6 vs. 42.6±4.4 for +/+ vs. +/−; P=.12, independent-sample *t* test). The decrease in adenoma number in $Rfc1^{+/-}Apc^{min/+}$ mice, compared to $Rfc1^{+/+}Apc^{min/+}$ mice, was more striking when they were on CD (60.4±9.4 vs. 30.3±4.6 for +/+ vs. +/−; P=.03, independent-sample *t* test). It is possible that dietary intervention after weaning ablated the decrease in adenoma number resulting from *Rfc1* deficiency. Low dietary folate is an important risk factor for colorectal cancer in human populations [3,4]; despite the fact that polymorphism in the folate-metabolizing enzyme MTHFR may be protective, this decreased risk is observed only when folate status is adequate [1]. Low dietary folate overcomes any potential benefit from genetic variants in human populations and may also be a risk factor under certain conditions in these animal studies (as discussed for *Mtr* mice below).

A recent study investigated the effect of *Rfc1* on azoxymethane-induced ACF formation. In this model, certain subtypes of ACF are thought to represent colorectal cancer precursors [36]. They showed that *Rfc1* status did not affect either the absolute number of ACF or the incidence of adenocarcinoma in the colon [24]. However, when crypt multiplicity was considered, they found that $Rfc1^{+/-}$ mice had a higher number of ACF, with more than one crypt per focus (larger ACF). The disparity between this finding and our own may be attributable to the model (genetic *Apc* disruption vs. chemical carcinogen) or to localization (small intestine vs. colon). The $Apc^{min/+}$ mouse model is associated primarily with small intestinal tumors, and tumor formation is initiated early since the mutation is present in the germline. The effect of *Rfc1* mutation that is also present in the germline may have consequences different from those of dietary deficiency or carcinogen treatment administered to older animals.

The genetic deficiency of *Mtr* did not elicit the same response as that of *Rfc1*. Although two epidemiological studies have suggested that individuals carrying an *Mtr* polymorphism have a decreased colorectal cancer risk [37,38], *Mtr* deficiency in this animal model did not affect tumorigenesis. $Mtr^{-/-}$ mice are embryonically lethal, and heterozygous mice have decreased enzyme activity and a

moderate increase in homocysteine in female mice [25]. There is an alternative homocysteine remethylation pathway, catalyzed by betaine–homocysteine methyltransferase (BHMT), which may partially compensate for *Mtr* deficiency. BHMT is expressed in hepatic and renal tissues [39], but BHMT products (methionine or SAM) may circulate and offset a disturbance in the folate-dependent remethylation of homocysteine, as we suggested for *Mthfr* knockout mice and for humans with hyperhomocysteinemia [40]. The mixed genetic background of these mice is a limitation of this study and precludes the drawing of any definitive conclusions regarding the effect of genetic disruption of *Mtr* in this model.

In $Mtr^{+/+}Apc^{min/+}$ and $Mtr^{+/-}Apc^{min/+}$ mice, dietary folate deficiency significantly increased the number of adenomas. This increase is consistent with findings in epidemiological studies on low dietary folate in human populations, as mentioned above [3,4]. In these mice, folate deficiency caused a significant threefold increase in plasma homocysteine, a decrease in DNA methylation and an increase in intestinal cell apoptosis. Since hyperhomocysteinemia is known to increase SAH levels through a reversal of the SAH hydrolase reaction and since SAH is a potent inhibitor of SAM-dependent methylation reactions, marked hyperhomocysteinemia may be directly responsible for hypomethylation. In previous work, we found that tumor number in $Apc^{min/+}$ mice was positively correlated with levels of DNA hypomethylation [18].

The increase in apoptosis due to folate deficiency in $Mtr^{+/+}Apc^{min/+}$ and $Mtr^{+/-}Apc^{min/+}$ mice may also be related to hyperhomocysteinemia. Elevated homocysteine has been shown to increase the rates of apoptosis [41] and to induce the hyperproliferation of colon cancer cells [42]. High levels of apoptosis may lead to a high cell turnover, with decreased repair time and selection pressure for cells capable of transformation [14].

Folate deficiency results in lower levels of SAM [43], which is required for the de novo synthesis of choline, the precursor for betaine. Folate deficiency may produce a secondary choline deficiency, both by inhibiting its synthesis and by enhancing its depletion by activating BHMT-catalyzed homocysteine remethylation [44]. This may contribute to tumorigenesis since choline deficiency has been shown to induce apoptosis and to promote carcinogenesis [45].

In this study, we have shown that early disruption in folate metabolism, in the form of a germline mutation in the folate transporter *Rfc1*, resulted in fewer intestinal adenomas in $Apc^{min/+}$ mice predisposed to forming a large number of tumors. A later disruption in the form of dietary folate deficiency administered on weaning resulted in an increase in tumor number in a different strain with or without a mutation in *Mtr*. These findings recapitulate the complex relationship between folate metabolism and tumorigenesis and highlight the utility of genetic animal models for these types of studies.

Acknowledgments

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