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Susceptibility to intestinal tumorigenesis in folate-deficient mice may be influenced by variation in one-carbon metabolism and DNA repair☆

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Abstract

Low dietary folate is associated with increased risk of colorectal cancer. In earlier work, we showed that folate deficiency induced intestinal tumors in BALB/c but not C57Bl/6 mice through increased dUTP incorporation into DNA with consequent DNA damage. To determine whether strain differences between one-carbon metabolism and DNA repair pathways could contribute to increased tumorigenesis in BALB/c mice, we measured amino acids and folate in the normal intestinal tissue of both strains fed a control diet or a folate-deficient diet. We also determined the expression of critical folate-metabolizing enzymes and several DNA repair enzymes. BALB/c mice had lower intestinal serine (major cellular one-carbon donor), methionine and total folate than C57Bl/6 mice under both dietary conditions. BALB/c mice had higher messenger RNA and protein levels of three folate-interconverting enzymes: trifunctional methyleneTHF (5,10-methylenetetrahydrofolate) dehydrogenase–methenylTHF cyclohydrolase–formylTHF (10-formyltetrahydrofolate) synthetase 1, bifunctional methyleneTHF dehydrogenase–methenylTHF cyclohydrolase and methylenetetrahydrofolate reductase. This pattern of expression could limit the availability of methyleneTHF for conversion of dUMP to dTMP. BALB/c mice also had higher levels of uracil DNA glycosylase 2 protein without an increase in the rate-limiting DNA polymerase β enzyme, compared with C57Bl/6 mice. We conclude that BALB/c mice may be more prone to DNA damage through decreased amounts of one-carbon donors and the diversion of methyleneTHF away from the conversion of dUMP to dTMP. In addition, incomplete excision repair of uracil in DNA could lead to accumulation of toxic repair intermediates and promotion of tumorigenesis in this tumor-susceptible strain.

Keywords: Folate; Cancer; DNA damage; Repair; Metabolism; One-carbon donor

1. Introduction

Folate deficiency is believed to increase the risk for colorectal cancer [1–3] because adequate supply and interconversion of one-carbon units are critical for nucleotide synthesis, DNA integrity and methylation reactions (Fig. 1). Non-methyl folates 5,10-methylene-tetrahydrofolate (methyleneTHF) and 10-formyltetrahydrofolate (formylTHF) are required for thymidine synthesis and purine synthesis, respectively. The irreversible reduction of methyleneTHF to 5-methyltetrahydrofolate (methylTHF) by methylenetetrahydro-folate reductase (MTHFR) generates one-carbon units for the synthesis of methionine and *S*-adenosylmethionine (SAM), a univer-sal methyl donor [4]. 677C→T [5], a common variant of MTHFR in human populations, has been proposed as a risk factor for certain cancers when dietary folate is low [6]. Methionine synthase (MTR) generates methionine by transferring the methyl group from methylTHF to homocysteine; this reaction requires periodical activation by methionine synthase reductase (MTRR).

Serine is a major source of one-carbon units; it donates carbon to tetrahydrofolate (THF) to form glycine and methyleneTHF for nucleotide synthesis. This reversible reaction is catalyzed in the cytoplasm by serine hydroxymethyltransferase 1 (SHMT1) and in the mitochondria by serine hydroxymethyltransferase 2 (SHMT2) [7,8]. MethyleneTHF produced from this reaction can then be used by thymidylate synthase (TS) to form dTMP from dUMP. MethyleneTHF

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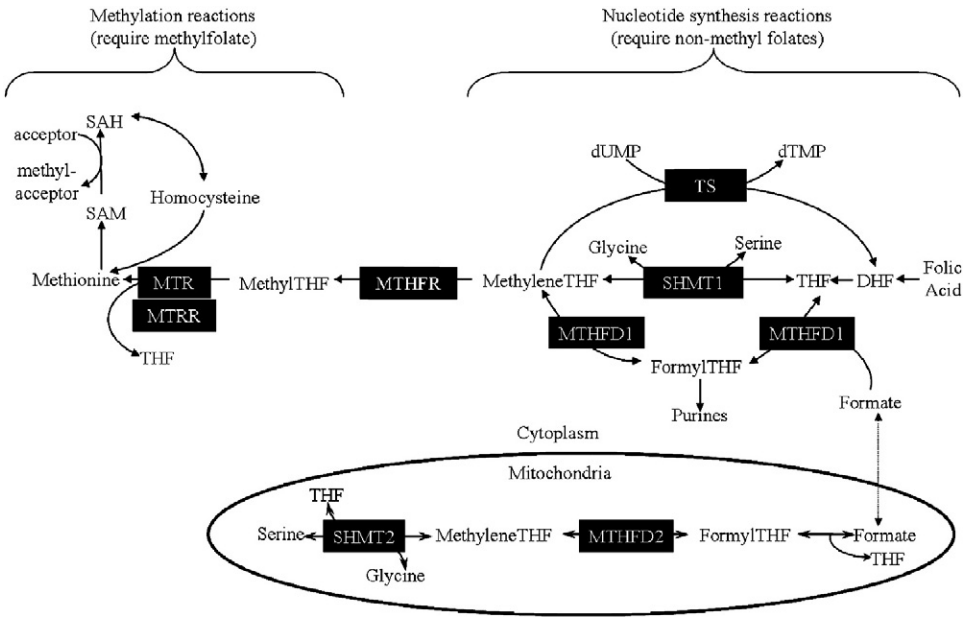

Fig. 1. Folate metabolic pathway. The oval represents the mitochondrial compartment. Enzyme names are indicated in boxes. SAH, *S*-adenosylhomocysteine.

can also be converted to formylTHF for purine synthesis by the trifunctional enzyme methyleneTHF dehydrogenase–methenylTHF cyclohydrolase–formylTHF synthetase 1 (MTHFD1) [9]. When there is an increased requirement for purines, such as during embryogenesis or tumorigenesis, the increased demand for formylTHF is met by the mitochondrial bifunctional enzyme methyleneTHF dehydrogenase–methenylTHF cyclohydrolase (MTHFD2), which uses methyleneTHF to generate formylTHF in the mitochondria [10,11]. The mitochondrial conversion of formylTHF to formate provides an exportable source of one-carbon units to the cytoplasm; the formate is then used by MTHFD1 to synthesize the additional formylTHF for purine synthesis [12,13].

Low dietary folate is hypothesized to lead to tumorigenesis through aberrant DNA methylation or disruption of the TS reaction. Insufficient methylTHF may result in global DNA hypomethylation, leading to chromosomal instability and inappropriate changes in gene expression [14]. Insufficient non-methyl folates for dTMP synthesis can lead to a build-up of dUMP, which can be misincorporated into DNA as dUTP, resulting in DNA double-strand breaks if not properly repaired [14]. Uracil misincorporated into nuclear DNA is removed primarily by the action of the UNG2 isoform of uracil DNA glycosylase [15]. Recently, an SNP in UNG was found to be associated with increased uracil content in DNA in a human population [16]. Thymine DNA glycosylase (TDG) excises uracil and thymine mispaired with guanine, which arise due to spontaneous hydrolytic deamination of cytosine and 5-methyl cytosine [17]. The removal of uracil results in an abasic site; the glycosylases remain attached, protecting the site, until the arrival of apurinic/apyrimidinic endonuclease 1 (APEX1). APEX1 cleaves the sugar–phosphate backbone of DNA, resulting in a DNA single-strand break that can be repaired by the short patch repair pathway. This involves removal of the 5′deoxyribose phosphate and insertion of the complementary base pair by DNA polymerase β (POLB), the rate-limiting step. The remaining nick in the DNA backbone is sealed by DNA ligase 3 [18].

In previous work, we showed that ~20% of BALB/c mice fed a folate-deficient diet (FDD) for 1 year developed intestinal tumors without induction by a carcinogen or genetic predisposition. There were more tumors in BALB/c mice that were heterozygous for a null allele in *Mthfr* (*Mthfr*$^{+/-}$) than in wild-type mice. In contrast, C57Bl/6 mice under the same conditions did not develop intestinal tumors [19,20]. We suggested that increased DNA damage due to increased DNA double-strand breaks might be an initiating event in tumorigenesis during folate deficiency, since the susceptible BALB/c strain had increased dUTP/dTTP levels and increased DNA double-strand breaks in the preneoplastic intestine, whereas the resistant C57Bl/6 strain did not show these changes [20]. We therefore examined the folate pathways in these strains in order to determine whether there were differences in the supply or utilization of one-carbon units that might lead to DNA damage. In addition, since it has been suggested that the BALB/c strain is susceptible to carcinogen-induced colorectal tumors [21,22] and may have decreased ability to rejoin radiation-induced double-strand breaks compared with C57Bl/6 mice [23], we questioned whether differences in repair of uracil-induced DNA damage could also play a role in differential susceptibility to intestinal tumorigenesis. The results of our study suggest that variation in folate-dependent gene expression, utilization of one-carbon units, and differences in repair of uracil-induced damage contribute to tumorigenesis in FD states.

2. Materials and methods

2.1. Mice

Animal experimentation was approved by the Montreal Children's Hospital Animal Care Committee. BALB/c and C57Bl/6 mice with and those without a null allele in *Mthfr* (*Mthfr*$^{+/-}$ and *Mthfr*$^{+/+}$, respectively) had been generated as reported, and tissue collection was performed as previously described [19,20].

2.2. Intestinal amino acids

Total cell lysates, prepared as described below for immunoblotting studies, were treated with dithiothreitol followed by sulfosalicylic acid, and the protein-free supernatant was analyzed for amino acids by ion-exchange chromatography [24].

2.3. Intestinal folate derivatives

Total folate and folate derivatives [methyleneTHF and THF, methylTHF, formylTHF and dihydrofolate (DHF)] in the intestine were measured by a ternary complex. In this assay, reduced folates are converted enzymatically to methyleneTHF, which then becomes part of a stable complex with TS and tritium-labeled FdUMP. Bound complex is separated from free ^{3}H-labeled FdUMP on a column and quantified by scintillation counting [25].

2.4. Quantitative RT-PCR

RNA was isolated from whole normal duodenum and quantitative RT-PCR was performed as previously described [19] using primers specific for *Apex1*, *MUTL homolog 1* (*Mlh1*), *MUTS homolog 2* (*Msh2*), *Mthfd1*, *Mthfd2*, *Mthfr*, *Mtr*, *Polb*, *Shmt1*, *Shmt2*, *Tdg*, *Ts* and *Ung*. Primer sequences and conditions are described in Supplementary Table 1. Target gene expression was normalized to glyceraldehyde-6-phosphate dehydrogenase (*Gapdh*). Results were normalized relative to the value for the control group, BALB/c CD (control diet) $Mthfr^{+/+}$.

2.5. Immunoblotting

Total cell lysates were prepared as described previously [26], quantified using Bio-Rad protein assay (Bio-Rad, Mississauga, Ontario, Canada) and stored at −80°C. Fifty micrograms of total cell lysate per sample was separated by SDS-PAGE, and immunoblotting was performed using antibodies directed against APEX1 (1:1000; Abcam, Cambridge, MA, USA), MTHFD1 (1:4000; a gift from Robert E. MacKenzie, McGill University), MTHFD2 (1:5000; also a gift from Robert E. MacKenzie), MTRR (1:5000; previously generated by our group [27]), POLB (1:1000; Abcam), SHMT2 (1:5000; a gift from Patrick Stover, Cornell University), TDG (1:5000; Ref. [28]) or UNG (2 μg/ml; Abcam). A dilution of anti-β-actin (Sigma, Oakville, Ontario, Canada) of 1:5000 was used as a control. A dilution of the appropriate HRP-conjugated secondary antibody of 1:5000 was used (donkey anti-rabbit, GE Healthcare, Baie-d'Urfé, Quebec, Canada; bovine anti-sheep or donkey anti-goat, Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Visualization was performed using the ECL Plus chemiluminescence kit (Amersham Biosciences, Baie-d'Urfé, Quebec, Canada). Quantity-One v.4.0.1 software (Bio-Rad) was used to quantify the appropriate bands. Immunoblotting results were normalized relative to the value for the control group, BALB/c CD $Mthfr^{+/+}$.

2.6. Enzyme activity assays

The MTHFR activity assay was performed in the reverse direction as previously described, by measuring the formation of ^{14}C-labeled formaldehyde-dimedone using ^{14}C-labeled methylTHF as the substrate [26]. The MTR activity assay was performed as previously described using ^{14}C-labeled 5-methylTHF, homocysteine, SAM and aqueous cobalamin to form ^{14}C-labeled methionine, which was separated by column chromatography and measured by scintillation counting [29].

2.7. Global DNA methylation analysis

Analysis of global DNA methylation was performed by thin-layer chromatography as reported previously [20].

2.8. Base excision assay

Mouse intestinal tissue samples were homogenized in lysis buffer [20 mM Tris–HCl (pH 7.8), 100 mM KCl, 2 mM EDTA, 5 mM β-mercaptoethanol, 1.5% Triton X-100, 10% glycerol and protease inhibitors]. Insoluble material was removed by centrifugation at 25,000*g* for 15 min (4°C), and the protein concentration of the lysate was determined by Bradford assay. Inclusion of 2 mM EDTA is essential to inhibit endonucleases and exonucleases. The base excision assays were performed as previously described [30] using asymmetrically radiolabeled duplex oligonucleotides containing a single G:T mispair [31]. Briefly, lysates (10 μg) were incubated for 8 h at 30°C with 8 ng of radiolabeled duplex oligonucleotide in a volume of 20 μl [28]. Excision of the mispaired thymine generates an abasic site that is then cleaved by alkali treatment to produce a diagnostic radiolabeled oligonucleotide. The samples were extracted with phenol/chloroform, precipitated with ethanol and then fractioned on 15% urea-polyacrylamide gel. The bands corresponding to the cleaved oligonucleotide and the unprocessed substrate were quantified by phosphorimaging and normalized to the signal for the BALB/c CD $Mthfr^{+/+}$ group.

2.9. Statistical analysis

Two-factor ANOVA was used to evaluate the effects of strain, diet, genotype and their interactions, followed by Tukey's *post hoc* test. For individual group comparisons, independent samples *t* test was used. SPSS for Windows (release 10.0.1) was used for analyses. Values are expressed as the mean±S.E.M. Differences were considered significant at $P<.05$.

3. Results

3.1. Differences in serine and methionine concentrations in intestine

We measured the concentrations of 28 free amino acids in the intestinal extracts of CD and FDD, $Mthfr^{+/+}$ and $Mthfr^{+/-}$, BALB/c and C57Bl/6 mice. Changes were observed for many amino acids (data not shown), but we elected to focus on serine (the major one-carbon donor for folate-dependent reactions) and methionine (the major one-carbon donor for SAM synthesis) (Fig. 2A). BALB/c mice had decreased intestinal serine and methionine concentrations compared with C57Bl/6 mice. These findings suggest that BALB/c mice may have fewer available one-carbon units for folate-dependent transfer reactions (*i.e.*, nucleotide synthesis and methylation).

Total intestinal homocysteine was below the levels of sensitivity of the assay. Plasma homocysteine levels have been previously reported [20].

3.2. Differences in total folate and distribution of folates in intestine

Intestinal extracts were used to measure total folate concentrations and several folate derivatives (Fig. 2B–D). With the methods available, methyleneTHF and THF could not be measured separately, nor could DHF and folate. We therefore present the data as percentages of non-methyl folate derivatives (methyleneTHF, THF, DHF, formylTHF and folate) and methylTHF, to assess the distribution of folates between nucleotide synthesis and the methylation cycle.

BALB/c mice had decreased levels of total folate (Fig. 2B, all folate derivatives) compared with C57Bl/6 mice. Within the C57Bl/6 strain, total folate pools were higher in the FDD group compared with the CD group, regardless of genotype. This increase may be due to increased one-carbon availability resulting from the elevated serine levels in the FDD C57Bl/6 mice, as shown in Fig. 2A. There was no dietary difference overall for total folate in the BALB/c strain. The decreased levels of total folate in BALB/c mice could result in a deficit of one-carbon units for nucleotide synthesis and DNA methylation reactions.

The percentage of non-methyl folates was lower and the percentage of methylTHF was higher in CD BALB/c mice compared with CD C57Bl/6 mice (Fig. 2C). However, when fed the FDD, the percentage of methylTHF (Fig. 2D) decreased significantly in BALB/c mice, whereas it increased in C57Bl/6 mice. These findings suggest that BALB/c mice may have lower methylation potential in the FD state.

3.3. Differences in messenger RNA levels of genes involved in folate metabolism

Quantitative RT-PCR was performed in the normal intestine to examine expression of critical genes involved in maintaining the levels of folate derivatives (Table 1). BALB/c mice had increased expression of *Shmt1*, *Shmt2*, *Mthfd1*, *Mthfd2*, *Mthfr* and *Mtrr* messenger RNA (mRNA) compared with C57Bl/6 mice, regardless of diet. Expression of *Mtr* was decreased in BALB/c mice compared with C57Bl/6 mice, but only when comparing within diet groups (CD or FDD). A dietary effect was observed for *Mthfd1* and *Mtrr* mRNA: both strains showed decreased expression in the FDD group compared with the CD group. No differences were found in *Ts* expression due to strain or diet. *Mthfr* genotype did not affect expression of any of the examined genes.

3.4. Immunoblotting or activity assays of enzymes in folate metabolism

To verify some of the expression changes observed at the mRNA level, we performed immunoblotting or enzyme activity assays. SHMT1 protein levels could not be detected in the intestine by immunoblotting. SHMT2 protein was also examined, but there were no differences due to strain or diet (data not shown). There are some data to suggest that SHMT2 is post-transcriptionally regulated [32].

MTHFD1 protein levels were examined in the intestine of 8 CD (4 $Mthfr^{+/+}$ and 4 $Mthfr^{+/-}$) and 12 FDD (6 $Mthfr^{+/+}$ and 6 $Mthfr^{+/-}$) BALB/c and C57Bl/6 mice. $Mthfr^{+/+}$ BALB/c mice had significantly increased MTHFD1 protein compared with $Mthfr^{+/+}$ C57Bl/6 mice,

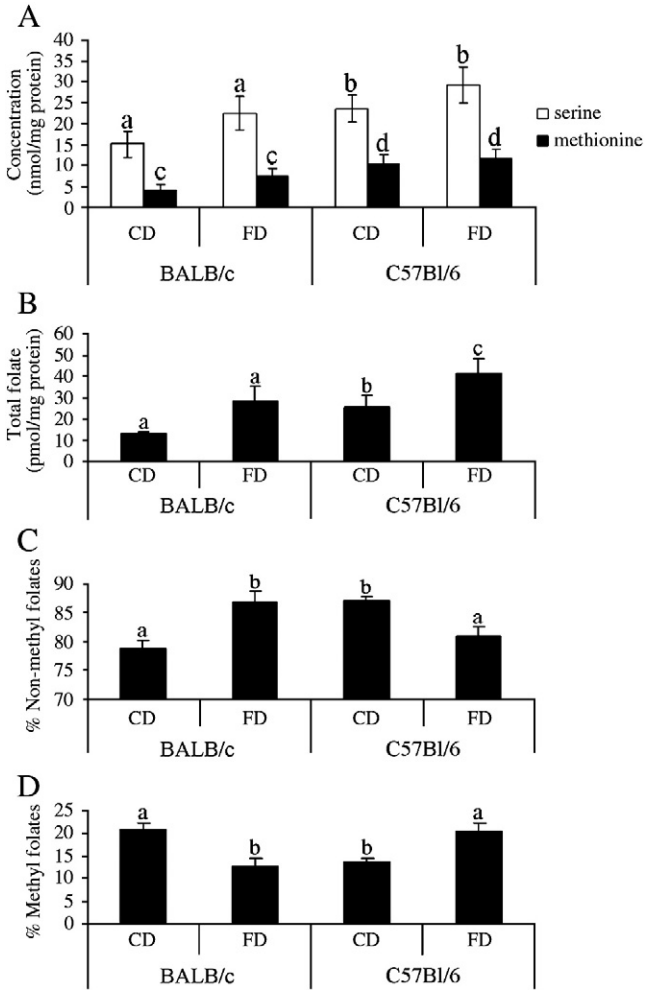

Fig. 2. Concentrations of serine and methionine (A) and total folate (B) and percentages of non-methyl (C) and methylfolate (D) derivatives in normal intestines of CD and FDD, BALB/c and C57Bl/6 mice. Genotypes were combined for the analyses shown in this figure. (A) Levels of serine and methionine in intestinal extracts. n=8 per group. Both serine and methionine were increased in C57Bl/6 mice compared with BALB/c mice. (B) Total folate levels in intestinal extracts. n=8 per group. C57Bl/6 mice had increased total folate levels compared with BALB/c mice. FDD C57Bl/6 mice also had increased total folate levels compared with CD C57Bl/6 mice. (C) Percentages of non-methyl folates (including methyleneTHF, THF, formylTHF, DHF and folate) in intestinal extracts. n=8 per group. CD C57Bl/6 mice had an increased percentage of non-methyl folates compared with CD BALB/c mice; the opposite was true for FDD C57Bl/6 mice compared with FDD BALB/c mice. FDD BALB/c mice had an increased percentage of non-methyl folates compared with CD BALB/c mice; the opposite was true when comparing CD and FDD C57Bl/6 mice. (D) Percentage of methylTHF in intestinal extracts. n=8 per group. CD C57Bl/6 mice had a decreased percentage of methylTHF compared with CD BALB/c mice; the opposite was true for FDD C57Bl/6 mice compared with FDD BALB/c mice. FDD BALB/c mice had a decreased percentage of methylTHF compared with CD BALB/c mice; the opposite was true when comparing CD and FDD C57Bl/6 mice. Values are expressed as the mean±S.E.M. In (A), "b">"a" (P<.05) by t test for strain difference, with diet groups combined. All other columns without a common letter differ (P<.05) by two-factor ANOVA.

regardless of diet (*Mthfr*$^{+/+}$ BALB/c=0.93±0.13; *Mthfr*$^{+/+}$ C57Bl/6=0.34±0.06; arbitrary units, relative to actin, P<.05). No significant change was observed for *Mthfr*$^{+/-}$ mice (*Mthfr*$^{+/-}$ BALB/c=0.96±0.19; *Mthfr*$^{+/-}$ C57Bl/6=1.18±0.19). We did not observe any significant differences due to diet in MTHFD1, but this negative finding may have been related to the high variability within each group. BALB/c mice had significantly increased MTHFD2 protein levels compared with C57Bl/6 mice, regardless of diet (Fig. 3A); these findings are consistent with the *Mthfd2* mRNA strain differences as mentioned above and shown in Table 1. Increased MTHFD2 expression in mitochondria would generate one-carbon units for transfer to cytoplasm as formate; increased MTHFD1 could drive these one-carbon units toward formation of methyleneTHF and formylTHF [12].

Measurements of MTHFR enzyme activity in normal intestine revealed that *Mthfr*$^{+/+}$ BALB/c mice had significantly increased MTHFR activity compared with *Mthfr*$^{+/+}$ C57Bl/6 mice in both dietary groups (Fig. 3B). FDD BALB/c *Mthfr*$^{+/+}$ mice also had increased MTHFR activity compared with CD BALB/c *Mthfr*$^{+/+}$ mice (Fig. 3B). The MTHFR activity results are consistent with the *Mthfr* mRNA strain differences, as mentioned above and shown in Table 1. Increased MTHFR activity would divert methyleneTHF from the TS reaction and potentially result in the increased dUTP/dTTP ratios that we previously reported for the BALB/c strain [20].

Since we observed differences in methionine levels and in the mRNA levels of the two genes directly involved in folate-dependent methionine synthesis (*Mtr* and *Mtrr*), we also assessed the activity and protein levels of these enzymes. The MTR enzyme assays revealed a decrease in MTR activity due to diet in the BALB/c strain, but there were no significant strain differences. These results are not consistent with the mRNA data in Table 1 and allude to post-transcriptional regulation of MTR, as previously reported [33,34].

The MTRR protein was detected in the intestine of 8/8 CD BALB/c mice and 2/8 FDD BALB/c mice but in only 2/7 CD C57Bl/6 mice and 0/8 FDD C57Bl/6 mice (Fig. 3C). This strain-dependent difference was observed even when the blots were exposed to film for 1 h or longer (as shown in Fig. 3C) and is consistent with the strain and diet differences observed in the *Mtrr* mRNA results (Table 1). Although the strain and dietary differences in MTR or MTRR allude to some interesting regulatory properties of these important enzymes, the results suggest that the decreased methionine levels observed in BALB/c mice are not likely due to the differences in levels of these two methionine biosynthetic enzymes.

3.5. Global DNA methylation analysis

We measured global DNA methylation in the normal intestine of the two strains by thin-layer chromatography. In our previous report, we had observed that BALB/c mice fed an FDD had decreased methylation compared with BALB/c mice fed CD; dietary differences in C57Bl/6 mice were not observed [20]. In that study, we could not directly compare the two strains as they were examined on different days. Here, we compared the two strains directly within each diet group. Consequently, the strain differences, but not the dietary differences within a strain, could be assessed in this study. The percent methylation did not differ between the two strains when they

Table 1
Summary of mRNA changes in genes involved in folate metabolism in normal intestines of CD and FDD, *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$, BALB/c and C57Bl/6 mice

	Gene	Strain difference[a]	Diet difference[b]
Non-methyl folate metabolism	*Shmt1*	3.7× ↑ in BALB/c vs. C57Bl/6	No
	Shmt2	5.7× ↑ in BALB/c vs. C57Bl/6	No
	Ts	No	No
	Mthfd1	1.9× ↑ in BALB/c vs. C57Bl/6	1.6× ↓ in FDD vs. CD
	Mthfd2	1.9× ↑ in BALB/c vs. C57Bl/6	No
	Mthfr	1.9× ↑ in BALB/c vs. C57Bl/6	No
MethylTHF metabolism	*Mtr*	3.8× ↓ in CD BALB/c vs. CD C57Bl/6[c]	No
		2.4× ↓ in FDD BALB/c vs. FDD C57Bl/6	
	Mtrr	2.0× ↑ in BALB/c vs. C57Bl/6	2.1× ↓ in FDD vs. CD

There were six mice per diet group per strain (three *Mthfr*$^{+/+}$ and three *Mthfr*$^{+/-}$). Arrows indicate a significant (P<.05) increase or decrease in expression for the comparison by two-factor ANOVA.
[a] Diet and genotype groups combined.
[b] Strain and genotype groups combined.
[c] P<.05 by t test.

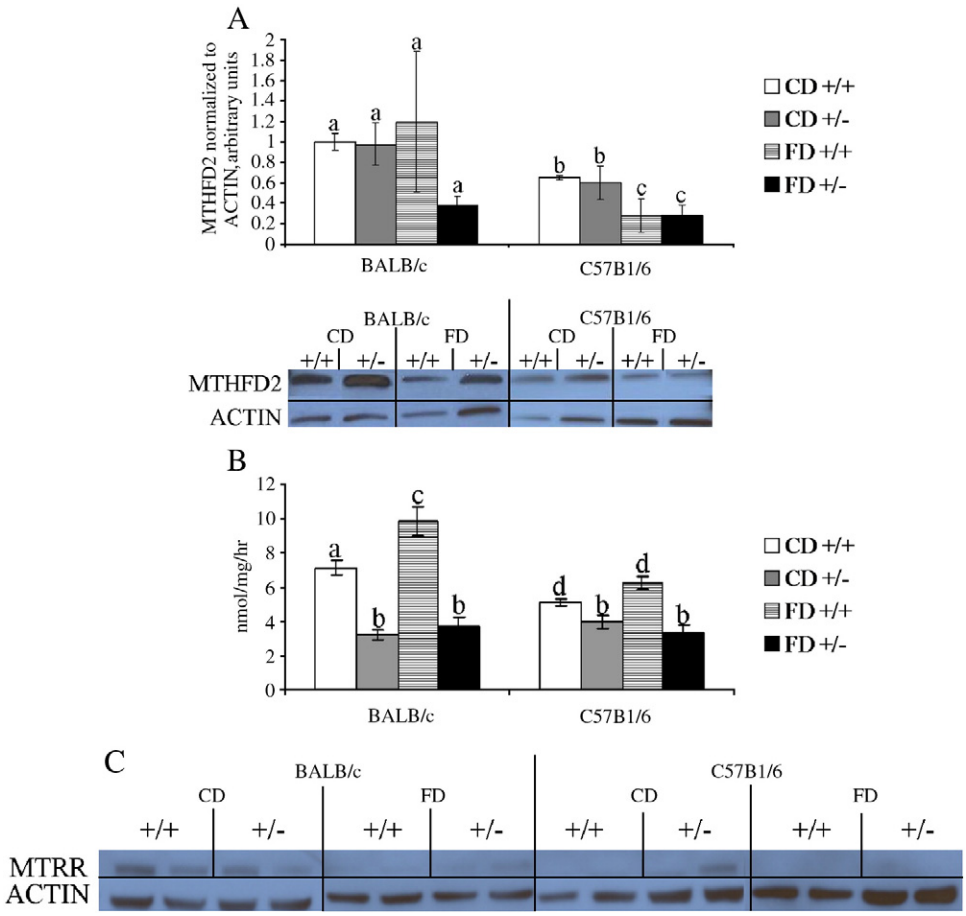

Fig. 3. Levels of immunoreactive protein or enzyme activity for MTHFD2 (A), MTHFR (B) and MTRR (C) in intestine from CD and FDD, *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$, BALB/c and C57Bl/6 mice. (A) Quantification of MTHFD2 immunoblots relative to actin (top panel) and a representative immunoblot (bottom panel) with actin as a control. There were a minimum of four mice per group. BALB/c mice had increased MTHFD2 protein compared with C57Bl/6 mice. (B) MTHFR enzyme activity. There were a minimum of three mice per group, except in the FDD *Mthfr*$^{+/+}$ BALB/c group, where $n=2$. *Mthfr*$^{+/+}$ BALB/c mice had increased MTHFR activity compared with *Mthfr*$^{+/+}$ C57Bl/6 mice in both CD and FDD groups. FDD *Mthfr*$^{+/+}$ BALB/c mice had increased MTHFR activity compared with CD *Mthfr*$^{+/+}$ BALB/c mice. *Mthfr*$^{+/+}$ mice had increased MTHFR activity compared with *Mthfr*$^{+/-}$ mice. (C) Representative MTRR immunoblots; 8/8 CD and 2/8 FDD BALB/c mice and 2/7 CD and 0/8 FDD C57Bl/6 mice showed a detectable band for MTRR, even when films were overexposed. Values are expressed as the mean±S.E.M. Columns without a common letter differ ($P<.05$) by two-factor ANOVA.

were compared in the same dietary groups (CD BALB/c, 55.9%±3.9%, compared with CD C57Bl/6, 58.1%±2.4%; FDD BALB/c, 64.2%±5.0%, compared with FDD C57Bl/6, 64.6%±2.4%).

3.6. Differences in mRNA levels of genes involved in DNA repair

Since BALB/c mice may have reduced capacity to repair radiation-induced double-strand breaks [23], we examined expression of several genes involved in the repair of DNA damage induced by folate deficiency (Table 2). Our analyses included several base excision repair enzymes (*Ung*, *Tdg*, *Apex1* and *Polb*) and mismatch repair enzymes (*Mlh1* and *Msh2*) (Table 2). Intestinal mRNA levels of *Mlh1* and *Mlh2*, two genes implicated in human colorectal cancer [35], did not differ between strains and were not affected by diet. In contrast, increased expression of *Ung* mRNA was observed in BALB/c mice compared with C57Bl/6 mice. BALB/c mice reduced expression of this mRNA in folate deficiency; C57Bl/6 mice did not exhibit dietary changes. Overall strain differences were not observed for *Tdg* mRNA. However, C57Bl/6 mice showed increased expression of this gene in response to folate deficiency, whereas BALB/c mice did not.

mRNA levels of *Apex1* (the next step in base excision repair after the action of DNA glycosylases [18]) were decreased in BALB/c mice compared with C57Bl/6 mice. Expression of *Polb* (the rate-limiting step in base excision repair [18]) was increased in BALB/c mice compared with C57Bl/6 mice (Table 2).

3.7. Immunoblotting or activity assays of enzymes involved in DNA repair

There were no differences in APEX1 or POLB protein levels as quantified from immunoblots, in contrast to the mRNA levels (APEX1: BALB/c=1.09±0.07, C57Bl/6=0.94±0.14; POLB: BALB/c=0.99±0.12, C57Bl/6=1.29±0.27; arbitrary units, relative to actin, n=a minimum of 12 with at least 6 each of CD and FDD mice per strain and at least 3 each of *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice per diet). These findings are consistent with reports suggesting that both APEX1 [36] and POLB [37] are regulated post-transcriptionally.

C57Bl/6 mice fed FDD tended ($P=.05$) to have increased TDG protein levels in the intestine compared with FDD BALB/c mice (Fig. 4A). To determine if there were any functional consequences of the altered levels of TDG, we measured G:T processing activity in intestinal tissue lysates derived from mice of different strain and diet groups. Significant differences were not observed between groups (Fig. 4B).

Immunoblotting for UNG (Fig. 4C) revealed striking differences between strains. BALB/c mice exhibited the larger molecular-weight nuclear isoform of UNG (UNG2, 34 kDa) in all samples, with no

Table 2
Summary of mRNA changes in genes involved in DNA repair in normal intestines of CD and FDD, *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$, BALB/c and C57Bl/6 mice

	Gene	Strain difference[a]	Diet difference[b]
Mismatch repair	*Mlh1*	No	No
	Msh2	No	No
Base excision repair	*Tdg*	No	3.2× ↑ in FDD vs. CD (C57Bl/6 only)
	Ung	3.1× ↑ in BALB/c vs. C57Bl/6	1.8× ↓ in FDD vs. CD (BALB/c only)
	Apex1	2.8× ↓ in BALB/c vs. C57Bl/6	No
	Polb	5.2× ↑ in BALB/c vs. C57Bl/6	No

There were a minimum of six mice per diet group per strain (at least three *Mthfr*$^{+/+}$ and at least three *Mthfr*$^{+/-}$). Arrows indicate a significant (P<.05) increase or decrease in expression by two-factor ANOVA.
[a] Diet and genotype groups combined.
[b] Strain and genotype groups combined.

evidence of a second isoform. C57Bl/6 mice exhibited lower levels of this isoform and instead showed a second band of high intensity in all samples; this band is consistent in size with the 31-kDa mitochondrial isoform (UNG1), which can also be localized to the nucleus in mice [38].

4. Discussion

Although inadequate dietary folate has been linked to colorectal cancer, the exact disturbances in one-carbon metabolism require elucidation. The availability of mouse models with differential susceptibility to intestinal tumor development following low dietary folate exposure provides an opportunity to identify genetic and non-genetic influences on tumorigenesis. In this study, we observed that the susceptible strain BALB/c had lower intestinal serine levels compared with the resistant strain C57Bl/6; serine is considered to be the major cellular one-carbon donor [4]. BALB/c mice also had lower total intestinal folate compared with C57Bl/6 mice. Lower serine and total folate in BALB/c mice would compromise dTMP synthesis and methylation reactions — two processes that are critical for folate-related tumorigenesis. The increased dUTP/dTTP ratios and decreased DNA methylation previously observed in BALB/c mice [20] are consistent with the overall low availability of one-carbon units. Although low dietary folate has been studied as a contributor to transformation, serine levels may warrant additional investigation.

We observed decreased intestinal methionine in BALB/c mice compared with C57Bl/6 mice. Methionine is the precursor of SAM; a decrease in methionine in BALB/c might result in a decreased ability to synthesize this important methyl donor. Our examination of the two ubiquitous major methionine biosynthetic enzymes (MTR, MTRR) suggests that the enzymatic capacity to synthesize methionine does not explain the different methionine levels in the two strains. The decrease in percent methylTHF in BALB/c mice during folate deficiency (in contrast to the increased percent methylTHF in C57Bl/6 mice fed an FDD) might contribute to the methionine differences between strains and affect critical methylation reactions. Methionine, as the precursor to SAM or to polyamines in rapid proliferative states, is an important intermediate in the tumorigenic process. Dietary studies in man have shown conflicting data on methionine levels; however, a recent meta-analysis that stratified data according to tumor sublocalization found that increased dietary methionine was associated with a decreased risk for proximal colorectal cancer in men [39]. Interestingly, recent data show that polyamine biosynthesis is a critical factor in determining sensitivity to folate depletion [40].

We reported a reduced ability of BALB/c (compared with C57Bl/6) to convert dUMP to dTMP, a reaction that requires methyleneTHF; this nucleotide imbalance results in uracil misincorporation into DNA and increased DNA damage [20]. Several observations in this study are consistent with those findings; our results suggest that there may be either reduced availability of methyleneTHF in BALB/c (decreased amounts of serine and total folate) or diversion of methyleneTHF toward other folate derivatives. In support of the latter case, BALB/c mice have increased mRNA and activity of MTHFR in both dietary conditions; the irreversible catalytic step performed by MTHFR converts methyleneTHF to methylTHF. Increased MTHFR may represent an attempt of the cellular machinery to restore methionine levels, since this enzyme is inhibited by SAM and methionine.

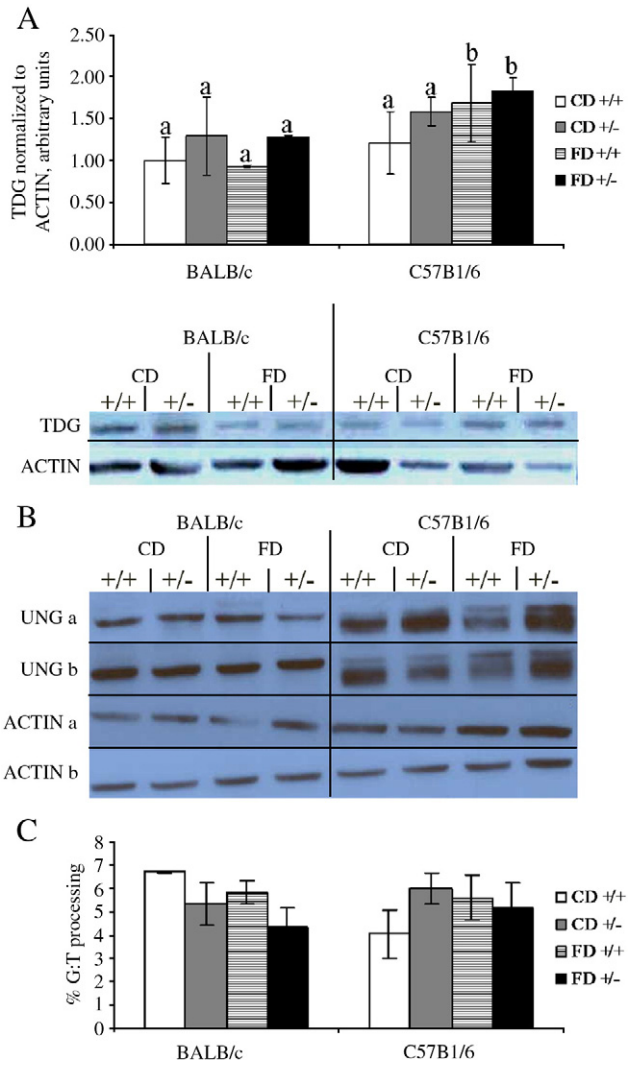

Fig. 4. TDG and UNG protein levels in intestines from CD and FDD, *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$, BALB/c and C57Bl/6 mice. (A) Quantification of TDG immunoblots relative to actin (top panel) and a representative blot (bottom panel) with actin as a control. n=4 per group. FDD C57Bl/6 mice had increased TDG protein compared with FDD BALB/c mice. (B) G:T processing activity in intestinal tissue. n=3 per group. There were no significant differences between groups. (C) Representative UNG immunoblots with actin as a control. n=4 per group. The "a" and "b" represent two different experiments. BALB/c mice show a unique UNG2 band at 34 kDa. C57Bl/6 mice show decreased amounts of the 34-kDa band with intense expression of the UNG1 isoform at 31 kDa. Values are expressed as the mean±S.E.M. Columns without a common letter differ (P=.05) by two-factor ANOVA.

Increased mRNA and protein were also observed for MTHFD2 and MTHFD1 in BALB/c mice. These activities could also divert methyleneTHF away from the TS reaction to formylTHF for purine synthesis, particularly since purine synthesis requires three one-carbon units, whereas dTMP synthesis only requires one [41]. MTHFD2 protein is only detected in tissues with a high demand for purine synthesis, such as tumors [42], and has not generally been detected in untransformed tissues [43]. This mitochondrial enzyme functions predominantly in the synthesis of formate for transfer to the cytoplasm and generation of one-carbon units for purine synthesis, in combination with MTHFD1; cells deficient in MTHFD1 are purine auxotrophs [44]. The shunting of one-carbon units toward purine synthesis in BALB/c mice may compromise dTMP synthesis, in addition to the limited serine, which is the major source of methyleneTHF.

If BALB/c mice accumulate more DNA damage due to accumulation of uracil and misincorporation into DNA, then their ability to repair this damage through the base excision repair pathway could be an important modifier of tumorigenesis. We did not observe any strain differences for APEX1 or POLB protein expression. This is noteworthy in the context of our observation that BALB/c mice appear to have increased expression of UNG2 (the higher molecular-weight UNG isoform). A study examining base excision repair in mice fed a diet without folic acid found that an increase in UNG2 levels without an increase in APEX1 or POLB resulted in an increase in single-strand breaks, compared with mice fed a folic acid-sufficient diet [45]. Therefore, the accumulation of these single-strand breaks in BALB/c mice, due to an imbalance in repair enzymes, could contribute to their enhanced susceptibility to tumorigenesis. In contrast, the decreased levels of UNG2 in C57BL/6 mice may not lead to an accumulation of DNA damage in that strain.

The strain differences in the nature of the UNG isoforms in the intestine are intriguing. Although the functions of these isoforms (encoded by a single gene) have not been clearly delineated, it has been suggested that UNG1 is the predominant isoform in resting cells in mice [46]. The increased amount of UNG1 in C57Bl/6 mice is consistent with a decreased proliferative state of this strain, as mentioned above in the discussion on MTHFD2. MTHFD2 is an enzyme that is seen in actively proliferating cells but is decreased in expression in C57Bl/6 mice compared with BALB/c mice.

Of additional interest is our observation that, in contrast to BALB/c mice, C57Bl/6 mice were able to increase the amount of TDG mRNA and protein in response to folate deficiency. Up-regulation of TDG expression may confer to C57Bl/6 mice an ability to repair both G:T mispairs arising from 5-methylcytosine deamination and other modified bases [47]. However, when we measured TDG repair activity in tissue lysates using an *in vitro* assay, we were not able to identify differences due to strain or diet. Nevertheless, considering the role of TDG in the detection of modified and mispaired bases on chromatin, it is plausible that increased levels would confer enhanced genome stability to FDD C57Bl/6 mice. TDG also appears to be an essential component of a multiprotein machinery that mediates CpG demethylation at target genes [48] and could therefore influence epigenetic regulation and contribute to the suppression of intestinal tumorigenesis in C57Bl/6 mice.

In conclusion, BALB/c mice may be more sensitive to intestinal tumorigenesis due to increased uracil-induced DNA damage and decreased ability to properly repair this damage. These mice have lower levels of total folate and serine in the intestine, as well as enhanced expression of enzymes that could limit the availability of methyleneTHF for dUMP conversion to dTMP. The increased amount of UNG2 in BALB/c mice, relative to C57Bl/6 mice, without a concomitant increase in POLB, may also result in the build-up of toxic intermediates and decreased capacity to effectively repair uracil-induced damage.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2010.07.015.

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