Azoxymethane-Induced Colon Carcinogenesis in Mice Occurs Independently of De Novo Thymidylate Synthesis Capacity\textsuperscript{1–3}

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Abstract

Folate metabolism affects DNA synthesis, methylation, mutation rates, genomic stability, and gene expression, which are altered in colon cancer. Serine hydroxymethyltransferase 1 (SHMT1) regulates thymidylate (dTMP) biosynthesis and uracil accumulation in DNA, and as such affects genome stability. Previously, we showed that decreased SHMT1 expression in Shmt1 knockout mice (Shmt1\textsuperscript{-/-}) or its impaired nuclear localization, as occurs in mice over-expressing an Shmt1 transgene (Shmt1\textsuperscript{tg+}), results in elevated uracil incorporation into DNA, which could affect colon cancer risk. We used these 2 models to determine the effect of altered SHMT1 expression and localization, and its interaction with folate insufficiency, on azoxymethane (AOM)-induced colon cancer in mice. Shmt1\textsuperscript{-/-} and Shmt1\textsuperscript{tg+} mice were weaned to a control or folate-and-choline-deficient (FCD) diet and fed the diet for 28 or 32 wk, respectively. At 6 wk of age, mice were injected weekly for 6 wk with 10 mg/kg AOM (w/v in saline). Colon uracil concentrations in nuclear DNA were elevated 2–7 fold in Shmt1\textsuperscript{-/-} and Shmt1\textsuperscript{tg+} mice. However, colon tumor incidence and numbers were not dependent on SHMT1 expression in Shmt1\textsuperscript{-/-} or Shmt1\textsuperscript{tg+} mice. The FCD diet reduced tumor load independent of Shmt1 genotype. In contrast, Shmt1\textsuperscript{tg+} mice exhibited a 30% reduction in tumor incidence, a 50% reduction in tumor number, and a 60% reduction in tumor load compared with wild-type mice independent of dietary folate intake. Our data indicate that uracil accumulation in DNA does not predict tumor number in AOM-mediated carcinogenesis. Furthermore, enrichment of SHMT1 in the cytoplasm, as observed in Shmt1\textsuperscript{tg+} mice, protects against AOM-mediated carcinogenesis independent of its role in nuclear de novo dTMP biosynthesis. 


Introduction

Folate insufficiency due to poor dietary intake or genetic polymorphisms that affect the expression or activity of folate-dependent enzymes have been associated with increased colorectal cancer risk, but causal mechanisms have remained elusive (1–3). Folate is a water-soluble B vitamin required for the de novo biosynthesis of purines, thymidylate (dTMP),\textsuperscript{7} and methionine (4). Folate deficiency can result in increased mutation rates, genome instability, and altered gene expression, characteristics of many cancers. Decreased de novo purine synthesis can result in genetic mutations, cell stasis, or death (5,6). Decreased de novo dTMP synthesis can result in increased rates of uracil misincorporation into DNA, leading to futile cycles of repair that ultimately lead to double-strand DNA breaks (7–9). Reduced cellular methylation potential due to impaired methionine and \textit{S}-adenosylmethionine (AdoMet) synthesis can result in altered DNA and histone methylation profiles and changes in gene expression and genome stability (10–12).

Serine hydroxymethyltransferase 1 (SHMT1), a folate-dependent enzyme, regulates de novo dTMP synthesis in the nucleus (13–19) (Supplemental Fig. 1). The de novo dTMP biosynthetic pathway includes SHMT1, methylenetetrahydrofolate dehydrogenase 1 (MTHFD1), dTMP synthase (TYMS), and dihydrofolate reductase (DHFR) (16). SHMT1, TYMS, and DHFR undergo coordinated ubiquitin-conjugating enzyme E2I (UBC9)-mediated sumoylation and translocation to the nucleus during the S phase of the cell cycle (16–18,20). In the nucleus, SHMT1 serves as an essential scaffold protein required for assembly of a metabolic complex that includes MTHFD1, TYMS, and

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\textsuperscript{3} Supplemental Tables 1 and 2 and Supplemental Figure 1 are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.

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\textsuperscript{5} Abbreviations used: AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; AOM, azoxymethane; DHF, dihydrofolate; DHFR, DHF reductase; dTMP, thymidylate; dUMP, deoxyuridylate; FCD, folate and choline deficient; MTHFD1, methylene-tetrahydrofolate dehydrogenase 1; NADPH, nicotinamide adenine dinucleotide phosphate; SHMT1, serine hydroxymethyltransferase 1; THF, tetrahydrofolate; TYMS, dTMP synthase; UBC9, ubiquitin-conjugating enzyme E2I.

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DHFR, and generates 5,10-methylene tetrahydrofolate and glycine from serine and tetrahydrofolate (THF). TYMS converts 5,10-methylene THF and deoxyuridylate (dUMP) to dihydrofolate (DFH) and dTMP. Finally, DHFR reduces DHF to THF in a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reaction.

In the cytoplasm, SHMT1 mediates the remethylation of homocysteine to form methionine (13,14). It does so by tightly binding and sequestering 5-methylTHF, the substrate of methionine synthase, and making it unavailable (21). Therefore, the presence or absence of SHMT1 in the cytoplasm regulates folate-dependent de novo methionine synthesis.

We leveraged 2 mouse models of altered SHMT1 expression and localization (described in Supplemental Table 1) to determine the effect of SHMT1 and its cellular localization on aoxosymethane (AOM)-induced colon cancer. The first is an Shmt1<sup>-/-</sup> knockout mouse model in which SHMT1 is reduced (hemizygous) or absent (homozygous) in both the cytoplasm and nucleus. Shmt1<sup>-/-</sup> mice demonstrate increased incorporation of uracil into nuclear DNA with minimal effect on cellular methylation capacity or purine synthesis, as well as enhanced adenomatous polyposis coli (Apc)<sup>min</sup>-mediated intestinal tumorigenesis (14,22). The second is a transgenic model of SHMT1 over-expression, the Shmt1<sup>tg+</sup> mouse, in which SHMT1 is over-expressed but does not translocate into the nucleus. Shmt1<sup>tg+</sup> mice demonstrate increased incorporation of uracil into nuclear DNA (19). In this study, we investigated the impact of altered SHMT1 expression and localization, resulting in reduced de novo dTMP synthesis and increased uracil incorporation in nuclear DNA, on susceptibility to AOM-induced colon cancer in Shmt1<sup>-/-</sup> and Shmt1<sup>tg+</sup> mice fed a control or folate-and-choline-deficient (FCD) diet.

Materials and Methods

Mice. Mice were specific-pathogen–free and maintained on a 12-h light/dark cycle at a controlled room temperature and humidity in accordance with standard use protocols and animal welfare regulations. Mice consumed food and water ad libitum. All study protocols were approved by the Institutional Animal Care and Use Committee of Cornell University and conform to the NIH Guide for the Care and Use of Laboratory Animals.

All mouse strains used in this study are described in detail in Supplemental Table 1. The generation of the Shmt1 null and Shmt1<sup>tg+</sup> transgenic mice has been described in detail elsewhere (14,19). Mice were genotyped by PCR using purified tail nuclear DNA (Qiagen). SHMT1 hemizygous null (Shmt1<sup>-/-</sup>) male mice were backcrossed a minimum of 10 generations onto the 129/SvJ inbred mouse strain. Male FVB/N (14,22). The second is a transgenic model of SHMT1 methylation capacity or purine synthesis, as well as enhanced for genotyping can be found in Supplemental Table 2.

Diets. Mice were weaned at 3 wk of age and randomly assigned to the control diet [AIN-93G containing 2-mg folic acid/kg and 2.5-g choline bitartrate/kg; Dyets (23)] or the FCD diet (AIN-93G diet lacking folic acid and choline; Dyets). Mice were maintained on the experimental diets for the duration of the study.

AOM treatment. Six-wk-old mice were injected intraperitoneally 1 time a wk for 6 wk with 10 mg/kg of AOM (w/v in saline; National Cancer Institute Chemical Carcinogen Reference Standards Repository) or vehicle only (saline). Seven to 10 male mice per experimental group were used for the Shmt1 null study. For the Shmt1 overexpression study, 10 or 11 mice per experimental group were used and each group consisted of 4 or 5 females and 5 or 6 males.

Tissue harvest. The feeding cycle was synchronized prior to tissue harvest to ensure that AdoMet concentrations reflected homocysteine remethylation capacity with minimal contributions from dietary methionine. Methionine intake can suppress the folate-dependent remethylation of homocysteine and thus mitigate any folate-dependent changes in metabolites of the methionine cycle. Food was removed 24 h prior to killing the animals. After 12 h, each animal was given 1 food pellet and the animals were killed 12 h later by cervical dislocation. Blood was collected into heparin-coated tubes by cardiac puncture. Plasma was isolated by centrifugation. Tissues including plasma, liver, and colon were flash-frozen in liquid nitrogen and stored at −80°C. The colon was flushed with cold PBS and opened longitudinally, and proximal, middle, and distal sections were flash frozen in liquid nitrogen.

Colon X-gal staining. Colonos from Shmt1<sup>tg+</sup> or Shmt1<sup>-/-</sup> mice were flushed with cold PBS, opened longitudinally, and stained with X-gal. Stained colon pieces were embedded in optimal cutting temperature (Tissue-Tek; Electron Microscopy Sciences) and frozen in isopentane cooled in liquid nitrogen. Cryosections that were 8-μm thick were affixed to plus-charged slides and examined using a light microscope.

Immunoblotting. Frozen colons (n = 3 or 4/group) were sonicated in lysis buffer [2% SDS, 100 mM, dithiothreitol, 60 mM Tris (pH 6.8)]. Total protein was extracted and quantified by the Lowry-Bensadoun assay (24). Proteins (40 μg/well for tissues) were separated on a 12% SDS-PAGE gel. Proteins were transferred at 4°C to an Immobilon-P polyvinylidene difluoride membrane (Millipore Corp.) using a Trans-blot apparatus (Bio-Rad). Membranes were blocked, probed with primary and secondary antibodies, and visualized as described previously (14). For SHMT1 detection, sheep anti-mouse SHMT1 antibody (25) was diluted 1:10,000, and rabbit anti-sheep IgG secondary antibody (Pierce) was diluted 1:20,000. For TYMS detection, affinity-purified sheep anti-human TYMS antibody (Abcam) was diluted 1:5000, and rabbit anti-sheep IgG secondary antibody (Pierce) was diluted 1:10,000. For actin detection, polyclonal rabbit anti-actin antibody conjugated to horseradish peroxidase (Abcam) was diluted 1:40,000.

Tumor assessment. The colon from the distal cecum to the anus was removed, flushed with cold PBS, opened longitudinally, and laid flat in lumen side up for examination using a dissecting microscope. Tumors were counted and their diameter measured by a pathologist-trained investigator who was unaware of the genotype of the specimen. Tumor load is a function of tumor number and area and was calculated as the total tumor area per mouse. Tumor incidence was determined as the presence of at least 1 tumor per mouse.

Determination of AdoMet and S-adenosylhomocysteine concentrations. Frozen tissues (n = 3–7/group for each study) were sonicated in 500 μL of 0.1 mM AcO buffer (pH 6), and protein was precipitated by adding 312 μL of 10% perchloric acid to each sample. Further processing and the measurement of AdoMet and S-adenosylhomocysteine (AdoHcy) were performed as described previously (14). AdoMet and AdoHcy values were normalized to total protein (24).

Colon folate concentration. Folate concentration in colon (n = 3–7/group for each study) was quantified using the Lactobacillus casei microbiological assay as described (14).

Uracil content in nuclear DNA. Nuclear DNA was extracted from 25–50 mg of tissue (n = 3–7/group for each study) using DNAse Tissue and Blood Kit (Qiagen), including an incubation with RNase A (Sigma) and RNase T1 (Ambion) for 30 min at 37°C. Ten μg of DNA was used to determine the uracil content of nuclear DNA, as described previously (14).

Statistical analyses. Two-factor ANOVA and Tukey’s post hoc test were used to determine the main effects and interactions of diet and genotype. Two-factor ANOVA and Tukey’s post hoc test were also used to determine a main effect of sex and genotype once it was established that diet did not
Colon of mice carrying the transgene (Shmt1flox/+), Endogenous Shmt1 transcriptional activity was detected by β-galactosidase activity (blue staining) in epithelial cells of the colon of mice with the targeting vector insertion (B) but not observed in wild-type mice (A). An inducible βgeo/Shmt1 expression vector was created to allow for detection of the Shmt1 transgene promoter activity by a lacZ reporter gene (C and D) (19) (Supplemental Table 1). Shmt1 transgene transcriptional activity was detected by β-galactosidase activity (blue staining) in a mosaic pattern in the epithelial and smooth muscle layer of the colon of mice carrying the transgene (Shmt1tg) but not in wild-type mice (Shmt1wt). C, colonic epithelium; L, lumen; lacZ, β-galactosidase; SHMT1, serine hydroxymethyltransferase 1; SM, smooth muscle.

Results

Effect of decreased Shmt1 expression on AOM-induced colon tumors and colon folate metabolism. The Shmt1flox/+ mouse strain carries an Shmt1floxβgeo targeting vector that was inserted into the Shmt1 gene by homologous recombination, as described by MacFarlane et al. (14) (Supplemental Table 1). The Shmt1floxβgeo cassette allows for the detection of endogenous Shmt1 transcription via the β-galactosidase (lacZ) reporter gene. Endogenous Shmt1 promoter activity was observed in colonic epithelial cells, indicated by β-galactosidase activity (blue stain) in Shmt1flox/+ mice (Fig. 1B) (14). β-galactosidase activity was not evident in the wild-type control tissue (Fig. 1A). We previously showed that the SHMT1 protein localizes to the cytoplasm of proliferating and mature enterocytes, as well as in the nucleus of progenitor crypt cells (22). SHMT1 is also endogenously expressed in multiple tissues, including the liver, kidney, and others (14).

A main effect of genotype on AOM-induced colon tumor number and incidence was not observed in Shmt1−/− mice (Fig. 2A). The FCD diet had no effect on tumor number; however, tumor load was significantly lower in mice fed the FCD diet compared with mice fed the control diet (Fig. 2B, diet effect) (P = 0.002), which is consistent with a role for dietary folate in DNA synthesis and tumor growth. Specifically, tumor load in Shmt1−/− mice fed the FCD diet was significantly lower than Shmt1+/+ mice fed the control diet (P = 0.002). Shmt1−/− and Shmt1+/− mice fed the control diet tended to have a smaller tumor load compared with Shmt1−/− mice fed the control diet (P = 0.07 and P = 0.09, respectively).

After 28 wk on the FCD diet, mice had significantly lower colon folate concentrations than mice fed the control diet (Table 1). We observed a significant genotype effect on colon folate such that Shmt1−/− mice had higher colon folate concentrations than Shmt1+/+ mice, similar to our previous observations of liver folate concentrations in Shmt1−/− mice (14). We did not observe differences in colon AdoMet, AdoHcy, or AdoMet:AdoHcy ratio among Shmt1−/−, Shmt1+/−, and Shmt1+/+ mice (Table 1), in contrast to observations made in the liver (14). Uracil in colon nuclear DNA was higher by 300–700% in Shmt1−/− mice compared with Shmt1+/+ mice (genotype effect, P < 0.05). Student’s t test (Table 1). There was no significant difference observed for uracil content in nuclear DNA in the colon.

Effect of reduced nuclear and increased cytoplasmic SHMT1 on AOM-induced colon tumors and colon folate metabolism. SHMT1-overexpressing mice carry an inducible βgeo/Shmt1 expression vector that allows for Crelox activation of the Shmt1 transgene (15). The βgeo/Shmt1 expression cassette also allows for detection and localization of the transgene expression, via the lacZ reporter gene. β-galactosidase activity (blue stain), indicative of Shmt1 transgene expression, demonstrated a mosaic expression pattern in the colonic epithelium and smooth muscle (Fig. 1C and D). Mice expressing both Cre (+) and the Shmt1 transgene, referred to as Shmt1tg, exhibited ~5-fold higher SHMT1 protein concentrations in the colon compared with Shmt1wt mice, but similar TYMS concen-

FIGURE 2. AOM-induced colon tumor number, and load in male Shmt1−/− mice after 28 wk on diet. Tumor number and incidence in Shmt1+/+, Shmt1−/−, and Shmt1+/− mice (A). Tumor incidence for each group is indicated above each bar. Tumor load in Shmt1+/+, Shmt1−/−, and Shmt1+/− mice (B). Values are means of ± SEMs, n = 7–10 mice per group. Two-factor ANOVA and Tukey’s post hoc test. P values are indicated. *Within genotype group, different from control diet (P ≤ 0.05). AOM, azoxymethane; D, diet; G, genotype; ns, not significant (P > 0.05); SHMT1, serine hydroxymethyltransferase 1.
trations (Fig. 3). The FCD diet was associated with a 150% increase in SHMT1 protein in Shmt1tg/+ mice, an effect that was not observed in Shmt11/−/− mice.

Shmt11/−/− mice demonstrated lower tumor incidence and numbers compared with Shmt1tg/+ mice (Fig. 4A). Ninety percent of Shmt11/−/− mice developed tumors, whereas only 64% of Shmt1tg/+ mice developed tumors (genotype effect: P = 0.04). Shmt1tg/+ mice had ~130% more tumors compared with Shmt11/−/− mice (genotype effect: P = 0.002). Similarly, we found that tumor load was ~200% higher in Shmt1tg/+ mice than in Shmt11/−/− mice (P = 0.002) (Fig. 4B). Of note, we observed a significant sex effect for tumor number (sex effect: P = 0.03) and load (sex effect: P = 0.006), such that male mice (genotype effect: P = 0.009) demonstrated higher Shmt1tg/+–dependent differences in tumor number than female mice (genotype effect: P = 0.08). Also, male, but not female, Shmt1tg/+ mice had significantly higher tumor loads than Shmt11/−/− male mice (genotype effect: P = 0.001). Diet did not significantly affect tumor incidence, number, or load.

For the analysis of folate-dependent metabolites in Shmt11/−/− mice, tissues from male mice were analyzed because they had a significant tumor phenotype. Genotype had no effect on colon folate concentration (Table 2). Colon folate concentration was >50% lower in mice fed the FCD diet than mice fed the control diet, independent of genotype (diet effect: P = 0.01). We did not observe any differences in colon AdoMet, AdoHcy, or the AdoMet:AdoHcy ratio among the mice (Table 2). We observed a significant 160–200% increase in uracil content in nuclear DNA in Shmt1tg/+ mice compared with Shmt11/−/− mice (genotype effect: P = 0.03) (Table 2). Uracil content in nuclear DNA was not significantly different in mice fed the FCD diet compared with mice fed the control diet.

Discussion

SHMT1 is required for de novo dTMP synthesis and also regulates methionine synthesis by mediating the remethylation of homocysteine. We previously showed that when SHMT1 expression is reduced in a hemizygous knockout mouse model, the resultant decrease in de novo dTMP synthesis capacity and the consequential increase in uracil incorporation into nuclear DNA increased AdoHcy-mediated intestinal tumorigenesis (22). Here, we used 2 models of altered SHMT1 expression and localization to determine the effect of reduced dTMP synthesis on AOM-induced colon carcinogenesis. Overall, we found that AOM-induced colon carcinogenesis occurs independent of decreased dTMP synthesis capacity and increased uracil incorporation in DNA.

Uracil incorporation into nuclear DNA can result in a futile cycle of DNA repair that leads to DNA double-strand breaks and genome instability (7–9), a proposed mechanism underlying folate-dependent colon cancer. We observed that an overall reduction in de novo dTMP synthesis capacity due to reduced SHMT1, as occurs in our Shmt11/−/− mice, resulted in increased uracil in nuclear DNA but not in any change in tumor incidence or number. In fact, the Shmt11/−/− mice fed the control diet had a lower tumor load than Shmt1tg/+ mice fed the control diet. Our data suggest that uracil-dependent DNA double-strand breaks likely do not play a significant role in tumor initiation because there was no relation between colonic DNA uracil content and tumor number, but that the breaks may inhibit tumor growth, as indicated by a reduction in tumor load. Similarly, the FCD diet was associated with ~85% fewer tumors in Shmt1tg/+ mice. Folate deficiency has been associated with the inhibition of tumor growth and progression in other studies (26,27), likely the result of reduced nucleotide synthesis and cell proliferation.

Perhaps counterintuitively, mice that overexpressed SHMT1 also demonstrated an increase in uracil incorporation in colonic nuclear DNA. However, we previously showed that Shmt11/−/− mice have lower SHMT1 and TYMS in the nuclear compartment and 50% lower nuclear dTMP synthesis, indicating that transgenically expressed SHMT1 does not translocate to the nucleus (19). Here, we found that despite a ~200% increase in uracil content in colonic DNA, Shmt11/−/− mice had a lower tumor incidence, fewer tumors, and lower tumor load than their wild-type counterparts, regardless of diet. Together, the data indicate that decreased nuclear localization of SHMT1, decreased rates of dTMP biosynthesis, and/or the associated increase in uracil-mediated genome instability are not associated with tumor initiation in the AOM model of colon cancer, but that they may be associated with tumor growth inhibition. These and other
data indicate that folate deficiency may inhibit the initiation and/or growth of AOM-induced colon tumors by limiting nucleotide synthesis, which could lead to reduced cell proliferation or the induction of apoptosis (26,28–30).

The results presented here are in contrast to our findings that decreased nuclear SHMT1 and elevated uracil in DNA increases Apcmin/+ decreased nuclear SHMT1 and elevated uracil in DNA increases synthesis, which could lead to reduced cell proliferation or the apoptosis in colonocytes (31,32). We proposed that increased MMTV integration site signaling pathway, and suppression of expression of a number of genes involved in the wingless-type (22).

In contrast, loss of heterozygosity of the Apc gene is not a necessary event for the initiation of AOM-induced colon cancer. Aberrant crypt foci, an early indicator for later tumor development in the AOM rodent model, do not demonstrate Apc mutations in rats (33,34). Also, Apc mutations are only observed in a small proportion (<20%) of AOM-induced adenocarcinoma in rats, and less in mice (33,34). Therefore, uracil incorporation and its potential effect on Apc gene mutations may have minimal impact on tumor initiation in the AOM model, which is supported by our data.

We observed an important difference in AOM-induced carcinogenesis between our 2 mouse models. An overall reduction in SHMT1 had little or no effect on tumorigenesis in Shmt1tg/+ mice; however, the overexpression and restriction of SHMT1 to the cytosol in Shmt1tg/+ mice was associated with reduced tumorigenesis. A primary function of SHMT1 is to support dTMP synthesis in the nucleus and prevent uracil accumulation into DNA, but it also tightly binds and sequesters 5-methylTHF in the cytosol, making 5-methylTHF unavailable for homocysteine remethylation (13,21). While liver AdoMet and the AdoMet:AdoHcy ratio are decreased in SHMT1 overexpressing mice, as would be expected if 5-methylTHF is sequestered (15), we did not observe significant changes in colon AdoMet or the AdoMet:AdoHcy ratio. However, the colon may compensate for limited 5-methylTHF availability by relying on alternative methyl donor sources for homocysteine remethylation, such as betaine, which could have unforeseen consequences on AOM-induced tumorigenesis.

The role of folate in carcinogenesis is complex, and it has been proposed as playing a dual role in colon cancer. Folate deficiency and inadequate folate metabolism caused by genetic polymorphisms have been associated with increased risk of colon cancer in humans (1,2), as well as with tumor initiation in rodents in most (28,35) but not all studies (36). In this study, we showed that uracil incorporation into nuclear DNA does not play a significant role in tumor initiation in the AOM model of colon cancer. However, our data suggest that impaired de novo dTMP synthesis leading to uracil accumulation in DNA may impair tumor growth. The mechanism by which enrichment of SHMT1 in the cytoplasm reduces tumor numbers in AOM-induced cancer remains to be determined.

**TABLE 2** Colon folate concentration, AdoMet, AdoHcy, AdoMet:AdoHcy ratio, and uracil content in DNA of male Shmt1tg/+ mice after 32-wk diet

<table>
<thead>
<tr>
<th></th>
<th>Control Diet</th>
<th>FCD Diet</th>
<th>P values</th>
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<tr>
<td></td>
<td>Shmt1wt/+</td>
<td>Shmt1tg/+</td>
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<tr>
<td>Folate, fmol/μg protein</td>
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<td>22.0 ± 0.805</td>
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<td>AdoMet, pmol/μg protein</td>
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<tr>
<td>AdoMet/AdoHcy</td>
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<tr>
<td>Uracil, μg/μg DNA</td>
<td>0.13 ± 0.022</td>
<td>0.36 ± 0.11</td>
<td>0.27 ± 0.064</td>
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</table>

1 Values are means ± SEMs; n = 3–8. *Different from Shmt1wt/+. P = 0.05 (within diet group). AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; FCD, folate and choline deficient; ns, not significant (P > 0.05).

2 P values are based on 2-factor ANOVA.
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Literature Cited