GNMT Expression Increases Hepatic Folate Contents and Folate-Dependent Methionine Synthase-Mediated Homocysteine Remethylation

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Glycine *N*-methyltransferase (GNMT) is a major hepatic enzyme that converts *S*-adenosylmethionine to *S*-adenosylhomocysteine while generating sarcosine from glycine, hence it can regulate mediating methyl group availability in mammalian cells. GNMT is also a major hepatic folate binding protein that binds to, and, subsequently, may be inhibited by 5-methyltetrafolate. GNMT is commonly diminished in human hepatoma; yet its role in cellular folate metabolism, in tumorigenesis and antifolate therapies, is not understood completely. In the present study, we investigated the impacts of GNMT expression on cell growth, folate status, methylfolate-dependent reactions and antifolate cytotoxicity. GNMT–diminished hepatoma cell lines transfected with GNMT were cultured under folate abundance or restriction. Folate-dependent homocysteine remethylation fluxes were investigated using stable isotopic tracers and gas chromatography/mass spectrometry. Folate status was compared between wild-type (WT), GNMT transgenic ($GNMT^{tg}$) and GNMT knockout ($GNMT^{ko}$) mice. In the cell model, GNMT expression increased folate concentration, induced folate-dependent homocysteine remethylation, and reduced antifolate methotrexate cytotoxicity. In the mouse models, $GNMT^{tg}$ had increased hepatic folate significantly, whereas $GNMT^{ko}$ had reduced folate. Liver folate levels correlated well with GNMT expressions ($r = 0.53$, $P = 0.002$); and methionine synthase expression was reduced significantly in $GNMT^{ko}$, demonstrating impaired methylfolate-dependent metabolism by GNMT deletion. In conclusion, we demonstrated novel findings that restoring GNMT assists methylfolate-dependent reactions and ameliorates the consequences of folate depletion. GNMT expression *in vivo* improves folate retention and bioavailability in the liver. Studies on how GNMT expression impacts the distribution of different folate cofactors and the regulation of specific folate dependent reactions are underway.

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INTRODUCTION

Different forms of folate serve as carriers of one-carbon units in DNA synthesis and biological methylation in mammals. The 10-formyl tetrahydrofolate is required for purine synthesis whereas the 5,10-methylenetetrahydrofolate is essential for pyrimidine synthesis. Among all forms of folates, the 5-methyl tetrahydrofolate (5-methyl-THF) is the most abundant that transfers the methyl group to the enzyme methionine synthase, generating methionine from homocysteine remethylation (1).

Glycine *N*-methyltransferase (GNMT, EC2.1.1.20) is an abundant liver protein that converts *S*-adenosylmethionine to *S*-adenosylhomocysteine while generating sarcosine from glycine. The GNMT reaction serves as an alternative pathway to regulate the *S*-adenosylmethionine to *S*-adenosylhomocysteine balance and availability of methyl group in mammalian cells (2). GNMT is also a major hepatic folate-binding protein (2,3) that binds to, and, subsequently, may be inhibited by 5-methyl-THF (2). The expression and function of GNMT have been investigated in human diseases (4–7) and mouse models (8–10). Downregulation of GNMT has also been reported in human hepatocellular carcinoma. Loss of heterozygosity within the *GNMT* gene in the liver tissues of hepatocellular carcinoma patients has been reported, and GNMT alteration appears to be an early event in human hepatocellular carcinoma (11). GNMT is commonly diminished in human hepatoma and hepatoma cell lines (4–10); hence it is believed to be a susceptibility gene and a potential tumor suppressor for human hepatoma (11). Individuals with mutant GNMT and GNMT knockout mice showed that inactivation

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of GNMT had significant impacts on methyl donor *S*-adenosylmethionine supplies as well as the *S*-adenosylmethionine to *S*-adenosylhomocysteine balance (8–10). Deletion of GNMT increased the susceptibility of liver cancer in mice (10).

The presence of GNMT in the liver and kidney implies that this protein could participate in gluconeogenesis (12). The function of GNMT in extra hepatic tissues is less clear. GNMT also is present in pancreas, prostate, intestinal mucosa, plasma and semen (12–17). The localization of GNMT in the exocrine cells in the above tissues suggests a potential role in secretion (12). GNMT serves as an alternative mechanism for utilizing *S*-adenosylmethionine that does not necessarily involve the methylation of physiologically important acceptors (12). The crystal structure of rat GNMT demonstrated two 5-methyl-THF binding sites located in the intersubunit areas of the tetramer (18). Each folate binding site consists of two 1–7 N-terminal regions of one pair of subunits and two 205–218 regions of the other pair of subunits, thus, each GNMT tetramer binds two folate molecules. The *N*-terminal fragments of GNMT need significant conformational freedom to provide access to the active sites for folate binding as well as for the inhibition by 5-methyl-THF (18). Although the binding between GNMT and 5-methyl-THF has been well characterized, the significance of maintaining optical folate status in human pathological conditions is less clear, and the specific impact of GNMT expression on hepatic methyl-folate-dependent reactions has not been elucidated. In recent years, stable isotope tracers have been widely utilized to elucidate how specific folate enzymes mediate and regulate the fluxes of one-carbon units among folate-dependent reactions (19–20). We demonstrated that transformed human lymphoblasts with reduced methylenetetrahydrofolate reductase (MTHFR) have advantages in *de novo* purine synthesis when folate is adequate, but they are more susceptible to *S*-adenosylmethionine depletion when folate is restricted (21). MTHFR, the enzyme that catalyzes the irreversible conversion of 5,10-methylene-tetrahydrofolate to 5-methyl-THF, is inhibited by *S*-adenosylmethionine (22–23). In mammals, the only known reaction that requires 5-methyl-THF is the synthesis of methionine from homocysteine (16). On the other hand, 5-methyl-THF is tightly bound to GNMT, and such binding inhibits GNMT (24). Both we and others have shown that GNMT deletion led to abnormally high elevations in *S*-adenosylmethionine and altered methylation status *in vivo* (8–10). Via its regulation in intracellular *S*-adenosylmethionine homeostasis and/or its tight binding to folate enzymes, it is plausible that GNMT can alter the availability of cellular folate cofactors and further affect folate-dependent reactions.

In the present study, we hypothesized that deletion of GNMT will result in low intracellular folate levels owing to decreased folate retention in the liver; and the loss of GNMT can cause folate deficiency specifically in those tissues originally expressing this folate binding protein. Alternatively, the tight binding between GNMT and 5-methyl-THF might reduce the availability or bioactivity of the enzyme-bound 5-methyl-THF, resulting in a decrease in the 5-methyl-THF-dependent homocysteine remethylation and methionine synthase expression. Impacts of GNMT expression on folate status were investigated both *in vitro* and *in vivo* using cell lines with and without GNMT transfection and by using genetic mouse models with GNMT transgene or GNMT disruption. The 5-methyl-THF dependent methionine synthesis metabolic fluxes were investigated in cell models with and without GNMT expression. The isotopic tracer studies enabled us to elucidate the impacts of GNMT overexpression and deletion on methyl-folate-dependent metabolic fluxes under various folate conditions.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The diminished GNMT activity in *Hep*G2 enabled us to study the impacts of restoring GNMT protein on folate metabolism. Establishing stable clones with and without GNMT was done in the human hepatoblastoma cell line *Hep*G2 by transfection and hygromycin (300 μg/mL) selection (25). A stable cell line, cotransfected with pGNMT and pTK-Hyg (Clontech, Palo Alto, CA, USA) plasmid DNAs, was used to represent cells with normal GNMT function (GNMT+). The other stable cell line that cotransfected with pFLAG-CMV-5 and pTK-Hyg plasmids was used to represent cells with diminished GNMT (GNMT–). GNMT expressions were confirmed by Western blot analyses in both cell lines.

To investigate the impacts of GNMT expression on folate status, intracellular folate contents and folate-dependent homocysteine remethylation fluxes were compared between GNMT+ and GNMT– cells under folate-adequate (regular αMEM media containing 2.2 μmol/L folic acid) and folate-restricted (10 nmol/L folinate) conditions. Cells were grown in α-MEM containing 10% (v/v) FCS, 0.12% $NaHCO_3$, penicillin (100,000 units/L), streptomycin (100 mg/L), amphotericin (0.25 mg/mL) and 5% CO_2 in an incubator at 37°C. The medium was replaced every 72 h. The treatments, including adequate folate, and low folate (10 nmol/L folinate), were treated for 144 h. Cellular folate, *S*-adenocylmethionine and *S*-adenosylhomocysteine concentrations were determined as described previously (21).

Stable Isotope Tracer Studies

GNMT-expressing *Hep*G2 cells (GNMT+) and the negative control (GNMT–) cells were cultured under conditions of folate abundance (100 nmol/L folinate) or mild folate restriction (10 nmol/L folinate) for 144 h. These levels were based on our previous experiments and were used to generate mildly low intracellular folate without affecting protein turnover significantly, and the tracer can still be taken (21). To investigate the impact of GNMT expression on the folate-dependent homocysteine remethylation fluxes, cells were plated in

a 60 mm dish at 30% to 50% confluence in the treatment medium supplemented with L-[2, 3, 3-$^2H_3$] serine (50% of total serine) combined with L-[5, 5, 5-$^2H_3$]-leucine (50% of total leucine) for 72 h. The folate-dependent homocysteine remethylation flux in the HepG2 cells was calculated as the relative enrichment in methionine + 1 from ^{13}C-serine (26).

Effects of GNMT Expression on Methotrexate-Induced Cytotoxicity

The impacts of GNMT expression on the antifolate drug cytotoxicity were investigated by treating these cells with various doses of methotrexate. GNMT+ and GNMT– cells were plated in a 24-well flat-bottomed culture plate in RPMI (3.5 × 10^4 cells/mL) and treated with methotrexate (50–1000 nmol/L) for 72 h. Cell survival was determined by the 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The absorbance was measured with a test wavelength at 570 nmol/L by sunrise enzyme-linked immunosorbent assay (ELISA) reader (Tecan, Salzburg, Austria). The IC_{50} values were calculated and compared between GNMT+ and GNMT– cells.

Methotrexate-induced cell apoptosis was then determined quantitatively by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit (Becton-Dickinson, Franklin Lakes, NJ, USA). GNMT+ and GNMT– cells were treated with low-dose (50 nmol/L) methotrexate for 72 h, harvested, washed with cold PBS and resuspended in Annexin binding buffer supplemented with Annexin V-FITC and PI. The mixture was incubated at 4°C in the dark for 10 min and immediately analyzed using the FACSCalibur system.

Genetic Mouse Models

The GNMT transgene mouse model ($GNMT^{tg}$) was established by microinjecting the Friend leukemia virus B (FVB)-fertilized eggs that had been transfected with the pEGFP-GNMT vector containing full-length human GNMT cDNA as described previously (27). $GNMT^{tg}$ had a significant increase in GNMT protein in the liver compared with the WT mice during the early stage. The GNMT knockout mouse model ($GNMT^{ko}$) was established by gene targeting disruption: the vector was constructed and transferred into embryonic stem cells, and then microinjected into blastocytes of C57BL/6 mice described previously (8). In comparison with the WT littermates ($GNMT^{wt}$[+/+]), the GNMT protein expression decreased by approximately 50% in the liver of heterozygous mice ($GNMT^{het}$[+/–]), and was undetectable in the nullizygous mice ($GNMT^{nul}$[–/–]). To investigate the impacts of GNMT expression on folate status, these genetic mouse models were bred and raised under specific pathogen-free conditions with temperatures between 20°–25°C at 50% humidity with a 12-h light–dark cycle throughout the study periods. Tail DNA was obtained for genotyping the offspring.

Conditions and Diets

All animal protocols were approved by the Institutional Animal Care and Use Committee of National Chung Hsing University. The animal experiments were conducted as follows: Study I was conducted to investigate the effects of GNMT overexpression on folate status. Liver folate concentrations were compared between $GNMT^{tg}$ and WT at wk 2, 5, 7, and 52 (1 year old). Study II was performed to examine the impacts of GNMT deletion on folate status using the $GNMT^{ko}$ model. Male $GNMT^{het}$ were mated with female $GNMT^{het}$ to obtain $GNMT^{wt}$, $GNMT^{het}$ and male $GNMT^{nul}$ littermates. Liver folate concentrations were compared among $GNMT^{wt}$, $GNMT^{het}$, and $GNMT^{ko}$ 1 month after weaning at 7 wks of age.

$GNMT^{tg}$ and $GNMT^{ko}$ mice received chow *ad libitum* throughout the periods in study I and II. To avoid lot-to-lot variability in the protein-bound folates from the chow diets, we further investigated folate status among tissues in $GNMT^{ko}$ mice that were fed the modified Clifford amino acid–defined rodent diet (Dyets, Bethlehem, PA, USA. We modified Dyets version of the Clifford/Kuory Folate Deficient Amino Acid Rodent Diet #517777 with RDA folate level. See http://www.dyets.com/510000.html). Protein source for rodent diets could have variable amino acid composition and uncertain quantities of protein bound vitamins. To minimize this effect and carefully control the vitamin content throughout the experimental period, investigators, including us, often choose amino acid–defined diets in folate metabolism and vitamin restriction experiments (28–29). In study III, parental mice and pups were fed an amino acid–defined diet containing 2 mg folate/kg diet (RDA for rodents) during mating, lactation, weaning and throughout the study periods. Folate status in the plasma, liver and spleen was compared between $GNMT^{wt}$, $GNMT^{het}$ and $GNMT^{ko}$. The offspring mice were euthanized after an overnight fasting at 7 wk of age. Plasma and tissues were harvested to investigate the effects of the GNMT defect on folate status and folate-dependent methionine synthase protein expression in the liver.

Determination of Plasma and Tissue Folate

Blood samples were collected in potassium-EDTA tubes and a plasma sample was isolated by centrifugation at 977*g* for 10 min at 4°C and stored at –80°C before analysis. Tissue folates were extracted in freshly prepared folate extraction buffer (30) containing 0.1% ascorbic acid using a polytron homogenizer. The supernatant fraction was collected and then treated with mouse serum conjugase to convert the folylpolyglutamates to their corresponding monoglutamate (31) at 37°C. The reaction was terminated at 121°C for 5 min. Folate concentrations were measured by a standard microbiological microtiter plate assay using *Lactobacillus casei* (31).

Determination of Methionine Synthase Expression

The expression of folate dependent protein methionine synthase (MTR) was determined by western blot. Approxi-

mately 0.03 g of liver was homogenized in ten volumes of RIPA buffer containing 0.1% (v/v) protease inhibitor cocktail Set 1 (Calbiochem, La Jolla, CA, USA). The tissue lysates were centrifuged at 18,360*g* for 30 min at 4°C and the supernatants were frozen in –80°C until analyses. The protein content was quantified by BCA Protein Assay (Pierce, Rockford, IL, USA). One-hundred micrograms of protein from each tissue were denatured in and then separated on 12% SDS-PAGE gels and transferred onto a PVDF membrane. After blocking with TBS containing 10% skim milk for 2 h, the membranes were incubated with the primary antibody, anti-MTR (1:1000) (Abcam, Cambridge, UK), β-actin (1:5000) (Millipore, California, USA) in TBS containing 5% skim milk at 4°C overnight. Membranes were washed 3x with TBS containing 0.1% Tween 20 (TBST) and then the membranes were covered with HRP-linked antigoat or antimouse IgG (1:5000) at room temperature for 2 h. The immunoblots were visualized by enhanced chemiluminescence kit (New England Biolabs, Beverly, MA, USA). To ensure equal protein loading, each membrane was stripped and reprobed with anti-β-actin antibody.

STATISTICS

All experiments were performed in triplicate. The statistical significance of difference in the means among groups was determined by analysis of variance (ANOVA). A Student *t* test was performed to detect any significant difference in the means between the control and treatment groups (SYSTAT, SPSS, Chicago, IL, USA). A *P* value < 0.05 was considered significant.

RESULTS

Effects of GNMT and Folate Supplies on Cell Growth, Protein Turnover, and Folate Concentrations

The doubling time, protein turnover, and intracellular folate concentrations were compared between GNMT– and GNMT+ cells under folate replete and deplete conditions (Table 1). Under adequate folate, GNMT expression tended to reduce the doubling time in *Hep*G2 cells by 1.5 to 2 h (mean doubling time was compared between GNMT+ and GNMT– by Student *t* test, *P* = 0.06 and *P* = 0.07 in Experiment I and II, respectively). When these cells were cultured under folate restriction, the difference in doubling time between GNMT+ and GNMT– became significant in both experiments (*P* < 0.001) (Table 1). In mild folate restriction (Experiment I, low folate for 144 h), doubling time increased significantly in GNMT– cells (*P* = 0.016) but not GNMT+ cells (*P* = 0.68). These data indicated that GNMT-expressing *Hep*G2 cells were less sensitive to folate depletion compared with cells with diminished GNMT expression. Growth retardation occurred in both cell lines when we prolonged folate depletion to 240 h, yet the doubling time of GNMT+ was significantly shorter than that of the GNMT– (*P* < 0.001) (Table 1). These results suggested that GNMT-expressing

Table 1. Effects of GNMT expression on cell growth, protein turnover, and folate contents.[a]

	Adequate folate[b]	Folate restriction[b]	% Change	*P* value
Cell growth[c]				
Experiment I[b]				
GNMT–[b]	55.3 ± 0.6	57.0 ± 0.5	**+3.0 ± 0.8**	**0.016**
GNMT+[b]	53.3 ± 1.2	53.6 ± 0.4	0.6 ± 0.7	0.68
P value	0.06	**<0.001**		
% Difference	−3.6 ± 2.3	**−5.9 ± 0.6**		
Experiment II[b]				
GNMT–	52.5 ± 0.6	**73.7 ± 0.3**	**+40.5 ± 0.5**	**<0.001**
GNMT+	51.1 ± 0.8	**69.5 ± 0.7**	**+36.1 ± 1.3**	**<0.001**
P value	0.07	**<0.001**		
% Difference	−2.7 ± 1.6	**−5.4 ± 0.9**		
Protein turnover[d]				
GNMT–	0.16 ± 0.01	0.14 ± 0.02	−7.6 ± 13.7	0.40
GNMT+	0.15 ± 0.01	0.15 ± 0.01	0.4 ± 1.6	0.93
P value	0.23	0.72		
% Difference	−4.9 ± 5.9	3.3 ± 1.6		
Folate (ng/10^6 cells)				
GNMT–	7.6 ± 0.4	2.0 ± 0.5	**−73.7 ± 6.9**	**<0.001**
GNMT+	11.4 ± 0.4	4.6 ± 0.5	**−54.5 ± 4.7**	**0.017**
P value	**<0.001**	**0.003**		
% Difference	**+49.5 ± 4.9**	**+130.7 ± 23.8**		

[a]All data are presented as means ± SD (n = 3). Each experiment was repeated at least twice. The *P* values were calculated by Student *t* test comparing two cell lines under each condition. The % change was calculated by comparing the mean with those under adequate folate. The % difference was calculated by comparing the mean with negative control cells under the same culture condition. The bolded data were statistically significant (*P* < 0.05).
[b]Cell lines and culture conditions. GNMT–: wild-type *Hep*G2 cells transfected with vector only were used as negative control. GNMT+: *Hep*G2 cells transfected with GNMT. Cells were cultured in a modified α-MEM medium under folate abundance (100 nmol/L folinate) or folate restriction (10 nmol/L folinate) for 144 h (Experiment I) or 240 h (Experiment II).
[c]Cell growth is presented as the doubling time (h) of each cell line under different folate conditions.
[d]Cells were cultured in α-MEM medium supplemented with L-(5,5,5-$^2H_3$)-leucine (200 μmol/L, 50% of total leucine) for 72 h. Protein turnover was estimated by the enrichments of leucine in the cellular proteins. Protein turnover was measured in cells cultured in low folate for 144 h.

*Hep*G2 cells are less sensitive to folate depletion.

Using L-[5, 5, 5-$^2H_3$]-leucine as the tracer we found that neither GNMT expression nor mild folate restriction significantly altered leucine enrichment in the cellular proteins, suggesting that total protein turnover did not change under these conditions (Table 1).

S-adenosylmethionine was decreased significantly (GNMT– versus GNMT+ cells = 3,195.8 ± 114.1 versus 2,313.4 ± 134.0 [pmol/mg protein], $P < 0.001$) and *S*-adenosylhomocysteine increased (GNMT– versus GNMT+ cells = 18.2 ± 1.2 versus 36.1 ± 0.8 [pmol/mg protein], $P = 0.003$) by GNMT expression. This observation was in agreement with the induced conversion of *S*-adenosylmethionine to *S*-adenosylhomocysteine by GNMT.

We discovered that GNMT expression specifically improved folate status under both folate repletion and mild folate restriction in *Hep*G2 cells. Folate concentration was 50% higher than that of the GNMT– under folate repletion and folate concentration was 131% higher than that of the GNMT– under folate restriction. Furthermore, folate concentration reduced by 74% in GNMT– whereas folate concentration decreased by 55% in GNMT+ (Table 1). These results proved that cellular folate contents in GNMT expressing cells are less depleted in response to folate restriction, and that GNMT expression can directly improve folate status regardless of medium folate conditions.

GNMT Expression Significantly Reduced Antifolate Methotrexate-Related Cytotoxicity

To investigate if the better folate status presented in GNMT+ actually protects these cells from antifolate toxicity, we further examined the effects of GNMT expression on the cytotoxicity induced by low dose methotrexate. Methotrexate inhibited cell proliferation in both cell lines, but GNMT+ cells were less sensitive to low dose (50–100 nmol/L) methotrexate.

The IC_{50} value of methotrexate was 550 nmol/L for GNMT+ and 450 nmol/L for GNMT– cells. Furthermore, restoring GNMT expression can protect HepG2 cells from apoptosis induced by low dose methotrexate treatments. At 50 nmol/L, MTX significantly induced apoptosis in GNMT– cells ($P = 0.011$ versus untreated cells), but not GNMT+ cells, as GNMT+ cells had a similarly low percentage of apoptotic cells as the untreated cells. These results demonstrated that restoring GNMT expression in *Hep*G2 cells can reduce methotrexate-induced cytotoxicity significantly (Figure 1).

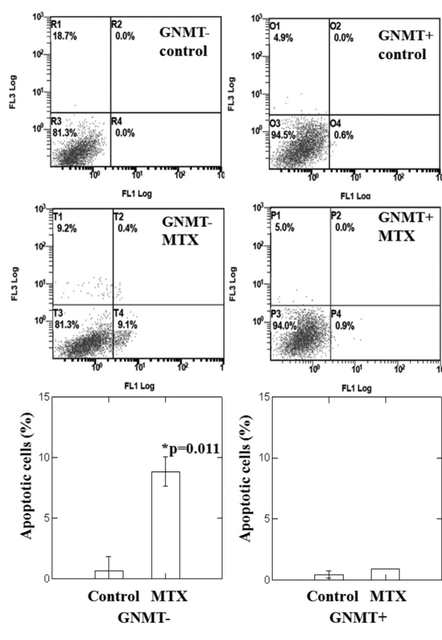

Figure 1. GNMT expression significantly reduced antifolate methotrexate-induced cytotoxicity *in vitro*.

GNMT Expression Significantly Improved Hepatic Folate Status *In Vivo*

In the mouse models, we discovered that GNMT expression significantly alters hepatic folate status *in vivo* (Figures 2–3). Normally, mice express GNMT protein in the liver at around 3 wks of age, around the time of weaning. The endogenous murine GNMT protein was either undetectable or detected at very low levels during the early stage of development before weaning. In contrast, the $GNMT^{tg}$ mice express human GNMT at birth (27). To investigate the impacts of GNMT expression on folate status, liver folate levels were compared between the

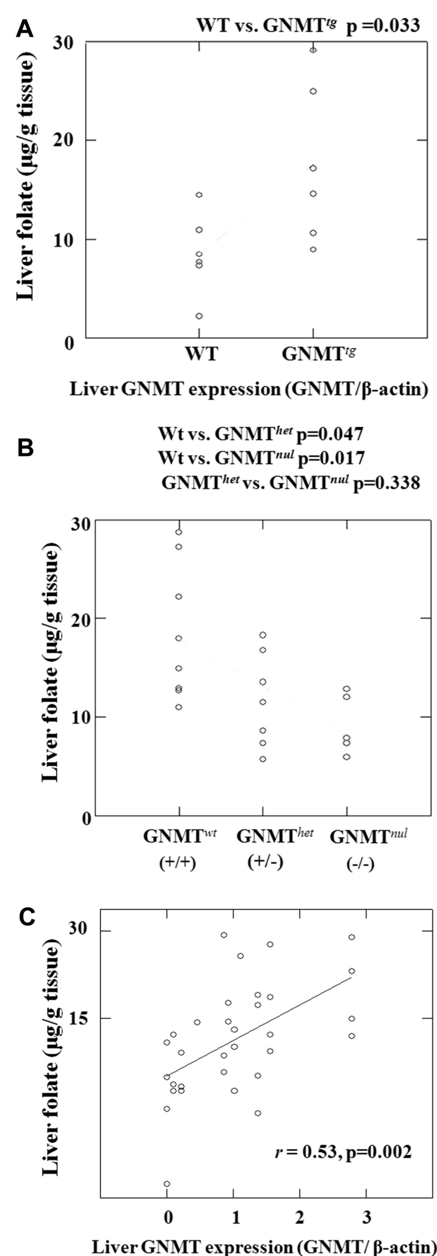

Figure 2. GNMT expression significantly alters hepatic folate status in (A) GNMT transgenic mice ($GNMT^{tg}$) (n = 6), compared with the wild-type (WT) littermates (n = 6); and in (B) the GNMT knockout mice ($GNMT^{nul}$ homozygous mice n = 5), compared with age matched littermates. $GNMT^{wt}$: wild-type (n = 8); $GNMT^{het}$: heterozygous (n = 7). (C) Liver folate concentrations were positively correlated with the liver GNMT expression levels when both models were combined ($r = 0.53$, $P = 0.002$, n = 32).

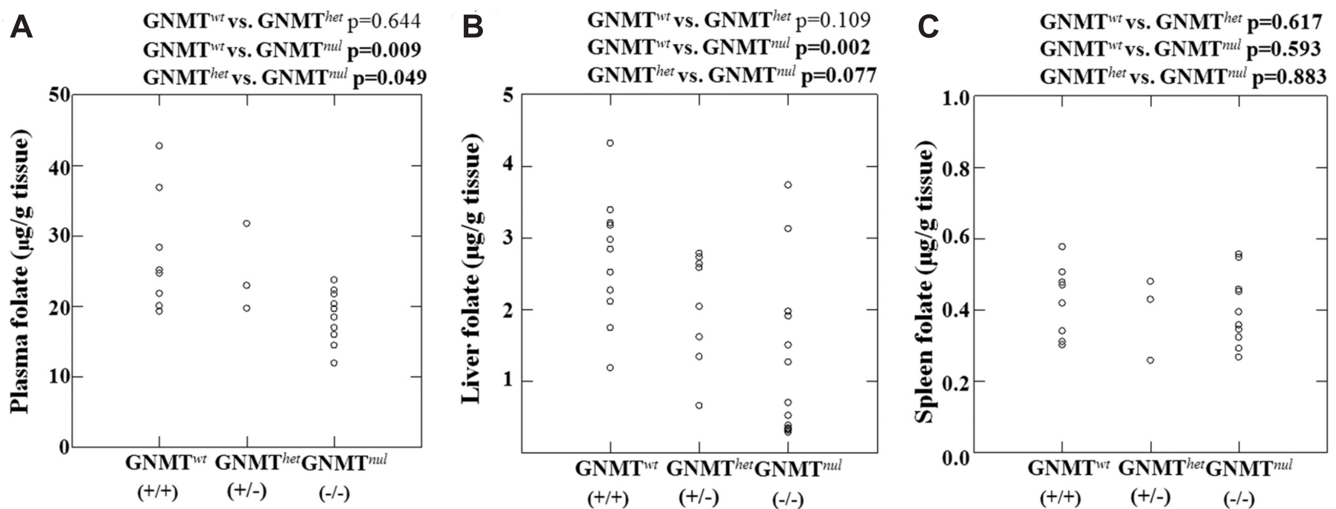

Figure 3. Tissue specificity of GNMT disruption on folate status. GNMT knockout significantly reduced folate concentrations (A) in plasma; (B) in GNMT expressing tissue liver; (C) but not in non-GNMT–expressing tissue spleen. GNMT wild-type (GNMT^{wt}), heterozygous (GNMT^{het}), homozygous (GNMT^{nul}) mice were bred from crossing heterozygous (GNMT^{het}) parents that received amino acid defined diet with 2 mg/kg folic acid and 0.1 % sulfathiazole. The offspring mice were fed the same diet after weaning.

WT littermates and GNMT^{tg} mice before and after weaning in Study I.

As we postulated, liver folate status is closely related and is comparable to the hepatic GNMT expression pattern. Compared with WT, hepatic GNMT levels were only significantly elevated during wks 1 to 3 in GNMT^{tg}. Consistently, liver folate levels significantly increased in GNMT^{tg} during this time period (mean ± SD in WT versus GNMT^{tg} = 8.5 ± 4.1 versus 17.5 ± 8.0 μg/g tissue, P = 0.033, n = 6 in each group) (Figure 2A). Furthermore, the hepatic GNMT expressions correlated well with liver folate concentrations before weaning (r = 0.615, P = 0.033, n = 12, data not shown). After weaning, GNMT expressions did not differ between GNMT^{tg} and WT. Consistent with this pattern, folate concentrations in the liver did not differ between WT and GNMT^{tg} at wk 5 (6.3 ± 2.4 versus 4.0 ± 2.2, P = 0.2), wk 8 (6.4 ± 1.1 versus 6.8 ± 3.0, P = 0.8), or up to wk 52 (6.3 ± 2.4 versus 4.0 ± 2.2, P = 0.7) (n = 5–6 per group at each time point, data not shown). Results from Study I supported our hypothesis that GNMT expression can improve folate status, presumably via increased hepatic folate retention *in vivo*. We have not observed any significant difference in body weight or food intake among the GNMT^{wt}, GNMT^{het} and GNMT^{ko} mice (data not shown). Therefore we suggest that the reduced folate levels observed in GNMT^{ko} mice was independent of lower folate intake.

GNMT Expression Induced Folate-Dependent Homocysteine Remethylation

To investigate whether the increased folate by GNMT overexpression can be bioavailable and utilizable for 5-methyl-THF-dependent methionine synthesis, the homocysteine remethylation fluxes were investigated further in stable isotopic tracer experiments under conditions of folate abundance and folate restriction. Under our experimental conditions, neither GNMT expression nor mild folate restriction significantly altered leucine enrichment from L-[5,5,5-$^{2}H_3$]-leucine tracer. On the other hand, GNMT expression significantly increased the relative methionine +1 enrichment from the ^{13}C-serine tracer, both in conditions of adequate folate and low folate (Table 2). These results supported our postulation that the methyl-folate-dependent homocysteine remethylation fluxes can be promoted by restoring GNMT function in cells with diminished GNMT. Taken together, we proved that GNMT expression increases cellular methyl-folate retention, and the retained folates are used effectively for the only biochemical reaction in which 5-methyl-THF participates.

GNMT Deletion Reduced Folate in the Liver

Next, we investigated whether GNMT plays an essential role in folate homeostasis by examining folate status in GNMT knockout mice (study II). Compared to GNMT^{wt}(+/+) (n = 8), mean liver folate concentrations were reduced by approximately 39% in GNMT^{het}(+/–) (P = 0.047, n = 7) and by 50% (P = 0.017, n = 5) in GNMT^{nul}(–/–) mice (Figure 2B). These data demonstrated that inactivation of GNMT directly led to loss of hepatic folate in a dose-dependent manner. Furthermore, when all data were combined from these mice expressing different levels of GNMT (GNMT^{wt}, GNMT^{tg} and GNMT^{ko}), liver folate concentrations correlated significantly with hepatic GNMT expression levels in these animals (R = 0.53, P = 0.002, n = 32) (Figure 2C). Mice in Study I and Study II were fed the chow diet throughout the study periods.

Table 2. Intracellular folate concentrations and folate dependent homocysteine remethylation fluxes in HepG2 cells with and without GNMT expression.[a]

	Leu +3 enrichment[b]	Ser +1 enrichment[b]	Met +1 enrichment[b]	Met +1 from ^{13}C-serine[b]
Adequate folate[c]				
GNMT–	0.161 ± 0.002	0.131 ± 0.004	0.007 ± 0.001	0.055 ± 0.001
GNMT+	0.153 ± 0.01	0.181 ± 0.014	0.028 ± 0.001	0.156 ± 0.016
P value	0.231	0.004	<0.001	<0.001
% Difference	–4.9 ± 5.9	+38.6 ± 10.5	+288.9 ± 13.6	+10.1 ± 1.7
Low folate[c]				
GNMT–	0.149 ± 0.022	0.138 ± 0.008	0.008 ± 0.001	0.056 ± 0.008
GNMT+	0.154 ± 0.002	0.173 ± 0.003	0.021 ± 0.001	0.118 ± 0.01
P value	0.725	0.002	<0.001	0.001
% Difference	3.2 ± 1.6	+25.2 ± 2.4	+163.2 ± 18.7	+6.2 ± 1.04

[a]All data are presented as means ± SD (n = 3). The *P* value, calculated by *t* test, compared two cell lines under each condition. The % difference was calculated by comparing the mean with negative control cells under the same culture condition. Cell lines. GNMT–: WT *Hep*G2 cells transfected with vector only were used as negative control. GNMT+: *Hep*G2 cells transfected with GNMT.
[b]Folate dependent homocysteine remethylation fluxes were calculated as the relative enrichments in methionine +1 from ^{13}C-serine. Cells were cultured in α-MEM medium supplemented with L-(^{13}C)-serine (237.8 μmol/L, 100% of total serine) combined with L-(5,5,5-$^{2}H_3$)-leucine (200 μmol/L, 50% of total leucine) for 72 h.
[c]Culture conditions. Cells were cultured in a modified α-MEM medium under folate abundance (100 nmol/L folinate) or mild folate restriction (10 nmol/L folinate) for 144 h.

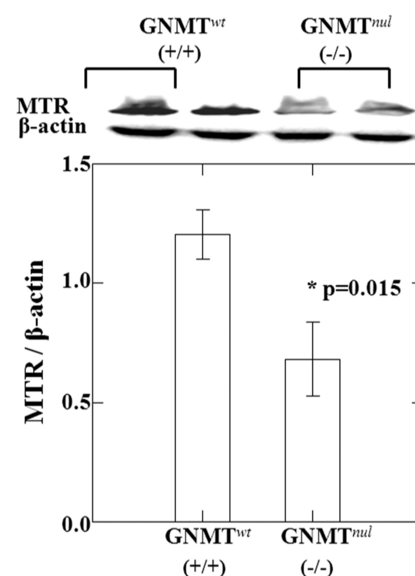

Figure 4. GNMT disruption significantly reduces hepatic MTR protein expression in mice. GNMT wild-type (GNMT^{wt}) and knockout (GNMT^{nul}).

Study III was conducted in GNMT^{ko} model–offspring mice on amino acid-based diet containing RDA folate contents throughout the life period. GNMT^{nul}(–/–) had significantly lower plasma folate concentrations compared with GNMT^{wt}(+/+) (P = 0.009) and GNMT^{het}(+/–) (P = 0.049) (Figure 3A). Consistently, GNMT^{nul}(–/–) has reduced folate contents compared with GNMT^{wt}(+/+) (P = 0.002) and GNMT^{het}(+/–) (P = 0.077) (Figure 3B). Plasma folate levels reflected folate status in the liver (r = 0.42, P = 0.066). In contrast, folate concentrations did not differ among different genotypes in non-GNMT–expressing spleen tissue (Figure 3C). Hepatic folate concentrations were significantly associated with GNMT expression in the liver. These results demonstrated that GNMT deletion had minimal impact on folate concentrations in the tissues without endogenous GNMT expression; and the impact of GNMT deletion on folate status was closely related to the tissue-specific expression patterns of this folate-binding protein. These results once again supported our postulation that normal hepatic GNMT expression not only improves folate status but also plays a crucial role in folate retention in the liver. Finally, the hepatic methylfolate-dependent enzyme MTR was reduced in GNMT^{nul}(–/–) mice (Figure 4).

DISCUSSION

In the present study, we demonstrated numerous findings. (a) Restoring GNMT in cells with diminished GNMT can improve intracellular folate status. (b) GNMT expression can increase 5-methyl-THF-dependent metabolic fluxes in GNMT–deficient cells. (c) GNMT ameliorates the growth retardation induced by folate depletion. (d) Expression of GNMT can protect HepG2 cells from low-dose methotrexate induced apoptosis. (e) *In vivo* GNMT expression improves folate status presumably owing to increased retention and bioavailability in the liver. (f) Destruction of GNMT *in vivo* specifically reduces hepatic folate and decreases methylfolate-dependent methionine synthase expression in the liver.

Our data demonstrated that GNMT disruption in mice resulted in reduction of the *5-methyltetrahydrofolatehomocysteine methyltransferase* (*MTR*) protein product in the liver. A recent study demonstrated that low dietary folate led to higher betaine demand and reduced MTR expression in mice (32). Our unpublished work also indicated that folate restriction causes low hepatic folate concentrations, decreased MTR protein and reduced folate dependent homocysteine remethylation fluxes in mice livers. These observations support the postulation that reduction in hepatic MTR protein seen in GNMT^{nul}(–/–) could result from hepatic folate depletion.

Folate status can determine the phenotypic expressions of folate metabolic enzymes and functions. One good example is found in the interactions between folate status and MTHFR C677T polymorphism in regulating different folate-dependent biochemical reactions. Previously, we demonstrated that human lymphoblasts with weaker MTHFR (homozygous of MTHFR C677T) have advantages in *de novo* purine synthesis when folate is adequate, but they are more susceptible to *S*-adenosylmethionine depletion when folate is restricted (21). In human colon and breast cancer cell models, MTHFR C677T mutation can induce cell-specific

alterations in DNA methylation (33) as well as uracil incorporations into the DNA (34). Both are potential molecular bases for cell- or site-specific cancer risk modification. MTHFR, a crucial enzyme that alters the distribution and utilization of different folate cofactors, is inhibited by *S*-adenosylmethionine. It is therefore plausible that hepatic GNMT expression affects the competition of folate cofactors between folate-dependent reactions via its regulation of intracellular *S*-adenosylmethionine homeostasis in the liver.

The inhibition of GNMT by 5-methyl-THF is an effective way to adjust and maintain the methyl group homeostasis in mammals. High dietary methionine intake leads to increased hepatic *S*-adenosylmethionine that inhibits MTHFR and lowers 5-methyl-THF levels. The reduced methylfolates alleviate the inhibition of GNMT, leading to more conversion of *S*-adenosylmethionine to *S*-adenosylhomocysteine (12). Normal GNMT function not only can protect animals against methionine toxicity, but also might assist cells to conserve methyl groups under methionine restriction. We recently discovered *in vitro* that GNMT expression does not exacerbate methyl group deficiency when the methionine supply is limited; instead it can protect cells from further hypomethylation induced by methionine depletion. Restoring GNMT expression in GNMT diminished cell lines is crucial in maintaining methyl group homeostasis via homocysteine transmethylation and transsulfuration kinetics (41). Adding to the current knowledge, results from the present study further demonstrated evidence that in GNMT–abundant tissue, that is, the liver, normal GNMT function is crucial for optimal folate status and hepatic-folate-dependent biochemical reactions *in vivo*. Our experiments in genetic mouse models demonstrated that inactivation of GNMT directly resulted in loss of hepatic folate in a dose-dependent manner, and normal GNMT expression is critical for folate retention in the liver. We suggest that these mechanisms may in part account for the protective effects of GNMT against liver cancer tumorigenesis and progression. The interactions and regulations among *S*-adenosylmethionine, GNMT, MTHFR and cellular folate are potential molecular mechanisms for cancer risk modification and are under investigation.

As the regulation of the key enzymes in the folate and methionine cycle is specific to gene, tissue or cell type (33), the impacts of GNMT expression on folate status and folate-dependent reactions may well differ among tissues. In our *in vitro* experiments, *Hep*G2 was chosen as it retains morphological and biological characteristics of normal human hepatocytes (35–37). Also, the endogenous GNMT expression in *Hep*G2 is almost undetectable, so we can easily distinguish the specific impacts derived from GNMT expression in this model. Furthermore, the diminished GNMT activity in these cells may represent the inactive GNMT protein in human hepatoma cells, thus enabling us to study the impacts of restoring GNMT protein on folate metabolism in these cells by transfecting GNMT. To examine the tissue-specific impacts of GNMT expression on folate status *in vivo*, folate status in the liver and the spleen were determined to represent tissues with and without normal GNMT expressions. Consistent with the expression pattern of GNMT, low folate status was found in GNMT^{ko} mice, and folate concentrations correlated with GNMT expression in the liver. Conversely, no difference was found in folate concentrations in the spleen between GNMT^{wt} and GNMT^{ko} mice.

Studies of mathematical modeling on hepatic one carbon metabolism indicated that as total folate decreases, the dissociations of numerous folate-enzyme complexes increase both the amount of active enzyme and additional free folate (38–39). These results implied that the enzyme-bound folates can be released under folate depletion. Results from our current study suggested that the GNMT expression can improve the retention of folate in the liver; and GNMT–bound 5-methyl-THF can be released readily and utilized for 5-methylfolate-dependent reactions when needed.

GNMT is a widely known folate-binding protein that is sensitive to inhibition by 5-methyl-THF polyglutamates (40). Here we provide novel *in vivo* and *in vitro* evidence that, by binding to 5-methyl-THF, GNMT may serve as a reservoir for intracellular folate that can be further utilized for folate-dependent reaction including homocysteine remethylation. We provide evidence that GNMT expression retains the folate in the liver; however, how GNMT can improve plasma folate levels remains to be investigated. We postulate that the reduced plasma folate concentrations in GNMT^{nul}(–/–) mice reflect poor folate status due to chronic folate loss by increased excretion. The urinary folate excretion is currently under investigation. Our present study demonstrated that intracellular folate status could be improved effectively when GNMT–diminished cells were transfected with GNMT, and restoring GNMT could ameliorate the consequences of folate restriction. Furthermore, GNMT could further ameliorate the cytotoxicity of antifolate treatment(s). Results from the present study provided direct evidence that GNMT expression in hepatocytes could improve folate status and that GNMT could play a crucial role in folate retention.

In conclusion, these mice and cell lines are feasible models for future investigations of the interactions between GNMT expression and folate metabolism in disease occurrence, progression or during antifolate treatments. Based on results from the present study, we suggest that normal GNMT function is important for reducing liver cytotoxicity and that normal GNMT function should be considered as a factor during antifolate chemotherapy or immunosuppressive treatments. As defective GNMT is commonly found in early hepatoma, the impacts of GNMT on folate metabolism may in part account for the protective role of GNMT against liver tumorigenesis. More studies are needed to determine whether these mechanisms par-

tially account for the protective role of GNMT against liver tumorigenesis. Studies on how GNMT expression impacts the distribution of different folate cofactors and the regulation of specific folate dependent reactions are underway.

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DISCLOSURE

The authors declare that they have no competing interests as defined by Molecular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

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