

# Folate Deficiency Alters Hepatic and Colon MGMT and OGG-1 DNA Repair Protein Expression in Rats but Has No Effect on Genome-Wide DNA Methylation

Susan J. Duthie<sup>1</sup>, George Grant<sup>2</sup>, Lynn P. Pirie<sup>1</sup>, Amanda J. Watson<sup>3</sup>, and Geoffrey P. Margison<sup>3</sup>

## Abstract

Folate deficiency is implicated in human colon cancer. The effects of feeding rats a folate-deficient diet for 24 weeks on DNA damage (8-oxo-7,8-dihydroguanine), DNA repair [*O*<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT) and 8-oxoguanine-DNA glycosylase (OGG-1) activity], and epigenetic parameters (genome-wide cytosine methylation and indices of cellular methylation status) were investigated. Relative to control diet, the folate-deficient diet resulted in significantly reduced levels of serum (~80%;  $P < 0.0001$ ), whole blood (~40%;  $P < 0.0001$ ), and tissue folate (between 25% and 60% depending on the tissue sampled;  $P < 0.05$ ); increased plasma total homocysteine (~35%;  $P < 0.05$ ); and decreased *S*-adenosylmethionine to *S*-adenosylhomocysteine concentrations (~11%;  $P < 0.05$ ). There was no significant change in the levels of 5-methyldeoxycytidine in liver or colon DNA, nor in the activity of liver DNA cytosine methyltransferase. However, there were significant increases in 8-oxo-7,8-dihydroguanine ( $P < 0.001$ ) in lymphocyte DNA and in levels of the DNA repair proteins OGG-1 (~27%;  $P < 0.03$ ) and MGMT (~25%;  $P < 0.003$ ) in the liver, but not in the colon. This may reflect the ability of the liver, but not the colon, to upregulate DNA repair enzymes in response to either elevated DNA damage or an imbalance in the nucleotide precursor pool. These results show that folate deficiency can significantly modulate DNA damage and DNA repair, providing mechanisms by which it plays a role in the etiology of human cancer. We speculate that the inability of colon tissue to respond to folate deficiency occurs in humans and may increase the potential for malignant transformation. *Cancer Prev Res*; 3(1); 92–100.

©2010 AACR.

## Introduction

Folates have a critical role in maintaining DNA stability by donating one-carbon moieties. High folate status is associated with a decreased risk of certain malignancies, including colorectal cancer (1, 2). However, an entirely protective role for folate against carcinogenesis has been questioned. Rodent studies report a reduction in early markers of colon cancer when folic acid is given before initiation of lesions (2–4). After initiation, folic acid increases carcinogenesis (5). Disturbingly, recent data indicate that an excessive intake of folic acid (from high-dose supplements or fortified foods) may increase human

cancers, including colon, prostate, and breast, by accelerating growth of precancerous lesions, but the dose and timing of such intervention is critical (6–9). However, on balance, evidence from the majority of human studies (retrospective, case control, and prospective) indicate that people who habitually consume the highest level of folate or who have the highest circulating folate have a 40% to 60% reduced relative risk of developing polyps or overt colorectal cancer (1, 2). A recent meta-analysis of prospective studies (10) reported a reduced risk for colorectal cancer in subjects with a high dietary folate intake compared with low intake (relative risk, 0.75; 95% confidence interval, 0.64–0.89). Existing evidence, therefore, indicates that increasing folate status in humans is genoprotective except perhaps in individuals with preexisting disease or who consume supranutritional levels of synthetic folic acid (6–9). Suboptimal folate status in humans remains widespread (11, 12).

How low folate increases cancer risk remains to be established, although multiple mechanisms have been postulated. Folate deficiency may induce epigenetic effects. Insufficient 5-methyltetrahydrofolate, by attenuating remethylation of *S*-adenosylhomocysteine (SAH) to *S*-adenosylmethionine (SAM) in the methionine cycle, leads to cytosine demethylation and global DNA hypomethylation. Genome-wide hypomethylation may

**Authors' Affiliations:** <sup>1</sup>Nutrition and Epigenetics Group, Division of Vascular Health and <sup>2</sup>Gut Immunology Group, Division of Gut Health, Rowett Institute of Nutrition and Health, University of Aberdeen, Aberdeen, United Kingdom and <sup>3</sup>Cancer Research UK Carcinogenesis Group, Paterson Institute for Cancer Research, University of Manchester, Manchester, United Kingdom

**Corresponding Author:** Susan J. Duthie, Nutrition and Epigenetics Group, Division of Vascular Health, Rowett Institute of Nutrition and Health, University of Aberdeen, Aberdeen AB21 9SB, United Kingdom. Phone: 44-1224-712751, ext. 2324; Fax: 44-1224-716629; E-mail: s.duthie@abdn.ac.uk.

doi: 10.1158/1940-6207.CAPR-09-0231

©2010 American Association for Cancer Research.

induce proto-oncogene activation and chromosomal instability, common features in human tumors (13, 14). Inadequate dietary folate results in decreased intracellular 5,10-methylenetetrahydrofolate, which retards conversion of dUMP to dTMP, leading to cellular thymidine depletion, uracil misincorporation into DNA, chromosomal breakage, and malignant transformation (15, 16). In addition to its effects on thymidine metabolism, folate deficiency affects purine biosynthesis by inhibiting 10-formyltetrahydrofolate-mediated production of adenosine and guanosine.

We speculate that folate insufficiency, in addition to inducing DNA hypomethylation and DNA damage, may accelerate genomic instability by impairing DNA repair. There is no question that cancer development is linked to compromised DNA repair. Classic examples of this are the defects in mismatch repair genes that are associated with hereditary nonpolyposis colon cancer (17) and mutations in nucleotide excision repair genes that underlie syndromes, such as xeroderma pigmentosum, with an associated increased risk of skin cancer (18). Moreover, in the general population, specific polymorphisms in DNA repair genes are associated with chromosomal abnormalities (19) and cancer risk (20).

To explore the effects of folate status on DNA repair capacity *in vivo*, we have determined in rats the differential effect of a prolonged folate deficiency on both the hepatic and colon activity of two DNA repair enzymes implicated in the development of human cancers (21–23). We have determined how folate deficiency affects concentrations of 8-oxo-7,8-dihydroguanine and the enzyme that repairs this lesion, 8-oxoguanine-DNA glycosylase (OGG-1). The potential for repair of alkylation damage has been assessed via quantitation of *O*<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT). In parallel, to

comprehensively assess the effect of folate deficiency on genomic stability, we have quantified global DNA methylation in these tissues.

## Materials and Methods

### Animals and diets

Amino acid-defined diets devoid of folic acid are an established means of predictably inducing folate deficiency (24, 25). Isoenergetic diets were formulated as described in Table 1. Mineral and vitamin mixes were in accordance with National Research Council recommendations (25) but were free of folic acid and the antibiotic succinyl sulfathiazole (Table 1). Succinyl sulfathiazole, which inhibits bacterial folate metabolism in the gut, is used in certain studies to induce severe folate deficiency (26, 27). Omission of antibiotic from the diet results in a mild to moderate folate deficiency, allowing the study to be carried out over a longer time period (24).

Male Hooded-Lister (Rowett strain) rats ( $n = 56$ ) were used. These animals are not genetically predisposed toward colon cancer, nor do they show signs of malignant transformation in response to long-term folate deficiency (25). They were weaned at 19 d, group housed, and given free access to control diet until they reached 95 to 100 g (40 d old). They were then individually housed on grid floors (to prevent coprophagy) and offered a fixed amount (12 g/d) of the same diet for 5 d.

At the beginning of the intervention (week 0), eight untreated rats (now ages between 6 and 7 wk) were killed by anesthetic overdose (halothane) and exsanguination via cardiac puncture. Blood was taken for plasma, erythrocyte, and lymphocyte preparation and tissues for folate analysis.

The remaining rats ( $n = 48$ ) were fed experimental diet [folate sufficient (F<sup>+</sup>) or folate-free (F<sup>-</sup>); 24 animals per diet] for up to 24 wk. We have shown previously that it is possible to detect a highly significant decrease in blood and tissue folate status using these numbers of animals in each group (25).

The rats were given a fixed amount of diet daily throughout the study: initially 12 g/rat/d, increasing to 15 g/rat/d after 1 wk, and to 16.5 g/rat/d from 6 wk onwards in accordance with the growth requirements of the animals. All food was eaten (data not shown). The amount offered was equivalent to 90% to 100% of normal free intake of semisynthetic diet. Water was available at all times, and the rats were weighed three times weekly.

All procedures were carried out in accordance with the requirements of UK Animals (Scientific Procedures) Act 1986.

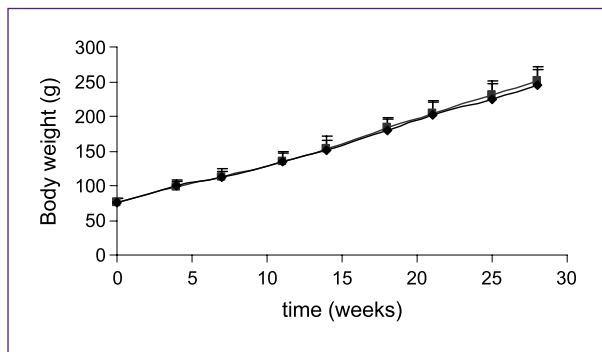
### Blood folate measured longitudinally throughout the intervention

The effect of intervention on blood folate status was measured longitudinally throughout the study. Samples were collected at weeks 0, 8, 16, and 24 after intervention. Whole blood (~0.5 mL) was collected from the tail vein, snap frozen in liquid nitrogen, and stored at -80°C or

**Table 1.** Composition of experimental diets (g/kg)

Diet	F <sup>+</sup> (folate-sufficient)	F <sup>-</sup> (folate-deficient)
Casein	112	112
Maize starch	382	382
Potato starch	100	100
Glucose	150	150
Corn oil	150	150
Minerals*	50	50
Vitamins*	50	50
Sodium silicate	0.2	0.2
L-Methionine	4	4
Folic acid mix	0.005	0
Choline chloride	2	2

\*Mineral and vitamin mixes were prepared as described (21), with the exception that folic acid was absent.



**Fig. 1.** The effect of folic acid deficiency on rat growth. Rats were fed either a folate-sufficient diet (F<sup>+</sup>; ♦) or a diet deficient in folic acid (F<sup>-</sup>; ■) for up to 24 wk. Points, mean body weight ( $n = 24$  per group); bars, SE.

centrifuged at  $2,400 \times g$  for 15 min at 4 °C. Plasma was aliquoted, snap frozen, and stored. Plasma and whole blood folate were determined in these samples by radioassay (25).

#### Blood and tissue collection after intervention

After 24 wk, the rats were killed by anesthetic overdose and exsanguination via cardiac puncture, and blood and tissues (brain, colon, kidney, liver, heart, and spleen) were collected. Whole blood from all animals was sampled by cardiac puncture and either snap frozen in liquid nitrogen and stored at -80 °C for analysis or centrifuged at  $2,400 \times g$  for 15 min at 4 °C. Plasma was aliquoted, snap frozen, heart, and stored. Lymphocytes from whole blood were prepared for folate analysis or quantification of DNA damage (described below). Cell number (lymphocytes) was determined using a hemocytometer before freezing. Brain, kidney, and spleen were snap frozen whole in liquid nitrogen for folate analysis. Colon from half the animals in each group ( $n = 12$ ) was perfused with ice-cold Tris-sucrose, opened flat, and dissected into proximal, transverse, and distal sections for folate, B12, DNA methylation, and SAM:SAH analysis. Liver from the same animals was perfused with ice-cold Tris-sucrose, blotted, and divided into individual lobes for analysis of the same biomarkers and DNA methyltransferase activity. Colon and liver from the remaining animals ( $n = 12$ ) were collected for DNA repair activity. All samples were snap frozen as above.

#### Oxidative DNA damage and misincorporated uracil

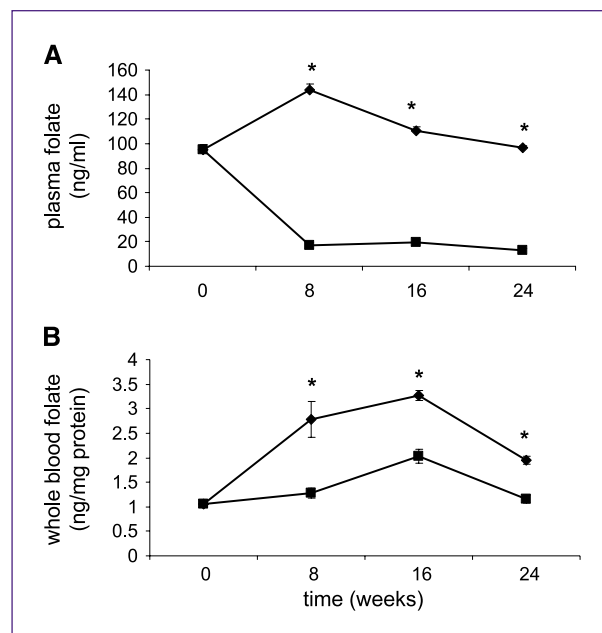
DNA damage was measured in rat lymphocytes at the end of the experiment. Whole blood (30  $\mu$ L) was resuspended in RPMI 1640 supplemented with 10% (v/v) FCS, underlain with LymphoPrep, and centrifuged at  $200 \times g$  for 4.5 min at 4 °C. The lymphocyte-rich buffy coat (>90% lymphocytes) was removed and analyzed immediately for DNA damage. Misincorporated uracil and 8-oxo-7,8-dihydroguanine were measured specifically using alkaline single-cell gel electrophoresis combined with either formamidopyrimidine glycosylase, the bacterial equivalent of mammalian OGG-1 protein (21), or uracil DNA glycosylase incubation, as described previously (25).

#### Oxidative and alkylation DNA repair protein activity (OGG-1 and MGMT)

Repair activities of OGG-1, which catalyzes the removal of mutagenic 8-oxo-7,8-dihydroguanine from DNA, and O<sup>6</sup>-alkylguanine-DNA alkyltransferase (MGMT), which repairs toxic, mutagenic, and carcinogenic O<sup>6</sup>-guanine damage, were determined in liver and colon scrapings by <sup>32</sup>P-labeled oligonucleotide cleavage assays. MGMT activity was determined by incubation of tissue sonicate (28) with a <sup>32</sup>P-labeled oligonucleotide containing O<sup>6</sup>-methylguanine within a *Pst*I recognition site attached via 3'biotin to the wells of streptavidin-coated plates. Repair of the O<sup>6</sup>-methylguanine by MGMT results in deprotection of the restriction site and, on incubation with *Pst*I, release of a short radiolabeled oligonucleotide fragment into the supernatant. MGMT activity is proportional to the amount of radioactivity present in the supernatant, quantified in a TOPCOUNT machine (Perkin-Elmer). OGG-1 activity was quantified in a similar way by measuring direct cleavage of an immobilized 8-oxoguanine-containing oligonucleotide (29).

#### Methyl group status, DNA methyltransferase activity, and genomic DNA methylation

Blood samples were collected after 24 wk on the folate-sufficient or folate-deficient diets, and plasma total homocysteine was measured by reverse-phase high-performance liquid chromatography using a DS30 Hcy Homocysteine



**Fig. 2.** The longitudinal effect of folic acid deficiency on plasma (A) and whole blood folate (B) levels in rats. Rats were fed either a folate-sufficient diet (F<sup>+</sup>; ♦) or a diet deficient in folic acid (F<sup>-</sup>; ■) for up to 24 wk. Points, mean ( $n = 10$  per group); bars, SE. \*,  $P < 0.0001$ , where significance refers to differences between F<sup>+</sup>- and F<sup>-</sup>-treated rats at each time point.

Assay kit in combination with a DS30 analyzer (Drew Scientific). Liver and distal colon SAM and SAH were measured in perchloric acid (PCA)-treated samples by high-performance liquid chromatography (30). Blood and tissue B12 levels were measured by radioassay (25). Liver nuclear proteins were prepared using a kit (EpiQuik Nuclear Extraction Kit I; Epigentek). DNA methyltransferase activity in these extracts was measured colorimetrically (EpiQuik DNA Methyltransferase Activity/Inhibition Assay kit; Epigentek). DNA was isolated from rat liver and colon using a Nucleospin C&T kit (Abgene Ltd.). 5-Methylcytosine levels were quantified by liquid chromatography-tandem mass spectrometry (LC MS/MS; ref. 31).

### Statistical analysis

Data are presented as mean  $\pm$  SE, with number of animals in parenthesis. Significant differences between treatment groups were analyzed by Students' *t* test using Statistical Package for the Social Sciences (version 13).

## Results

### Folate status

Rats were fed either a control diet containing folic acid (5 mg/kg diet) or a diet devoid of folic acid for up to 24 weeks. Folate deficiency had no significant effect on body weight (Fig. 1). No behavioral or pathologic abnormalities were observed in folate-deficient animals compared with controls.

In animals fed the folate-free diet, plasma folate significantly decreased to an 8-week low, which was maintained

for the duration of the study, resulting in  $\sim$ 80% of control levels by 24 weeks. There was an  $\sim$ 40% decrease in whole blood folate by 24 weeks (Fig. 2). Lymphocyte folate decreased 40% and uracil misincorporation increased 2-fold over the same period (Table 2; Fig. 3).

Tissue folate declined between 25% and 60% (depending on the organ) in animals fed the folate-deficient diet. Folate depletion was similar for all three colon regions ( $\sim$ 60%). Brain (25% of controls) and liver (31% of controls) were the organs least affected by dietary folate deficiency (Table 2).

### Oxidative DNA damage

Intracellular folate depletion in rat lymphocytes was associated with a significant increase in levels of the oxidized purine base 8-oxo-7,8-dihydroguanine (Fig. 3).

### DNA repair protein activity

Folate deficiency increased MGMT (25%) and OGG-1 (27%) activity in rat liver ( $P = 0.03$  and  $P = 0.003$ , respectively; Fig. 4A). In the distal colon, these DNA repair activities were unaffected by folate status (Fig. 4B), although there was a trend for decreased OGG-1 activity ( $P = 0.054$ ) in folate-deficient tissue.

### Methyl group status, DNA methyltransferase activity, and genomic DNA methylation

Folate deficiency increased plasma homocysteine ( $\sim$ 25%;  $P < 0.02$ ; Table 3). There was no effect of folate deficiency on B vitamin status (plasma, lymphocyte, liver, or colon). The ratio of hepatic SAM to SAH was significantly decreased in rats fed a folate-free diet for 24 weeks

**Table 2.** Folate concentrations in lymphocytes and tissues from rats fed a folate-supplemented (F<sup>+</sup>) or folate-deficient (F<sup>-</sup>) diet for 24 wk

	Folate (ng/mg protein)		Depletion (%)
	F <sup>+</sup>	F <sup>-</sup>	
Lymphocyte (ng/10 <sup>5</sup> cells)	0.45 $\pm$ 0.08 (8)	0.27 $\pm$ 0.03* (8)	39
Liver	136.2 $\pm$ 6.9 (10)	93.9 $\pm$ 16.3* (10)	31
Colon (ascending)	25.7 $\pm$ 4.2 (10)	11.9 $\pm$ 1.1 <sup>†</sup> (10)	54
Colon (transverse)	27.4 $\pm$ 3.2 (9)	10.5 $\pm$ 1.6 <sup>‡</sup> (10)	62
Colon (descending)	24.6 $\pm$ 1.9 (10)	9.9 $\pm$ 1.8 <sup>§</sup> (10)	60
Spleen	16.5 $\pm$ 2.9 (10)	7.9 $\pm$ 1.1 <sup>†</sup> (10)	52
Kidney	58.6 $\pm$ 7.5 (10)	26.6 $\pm$ 2.7 <sup>‡</sup> (10)	55
Brain	20.4 $\pm$ 0.5 (6)	15.3 $\pm$ 2.2* (6)	25
Heart	9.2 $\pm$ 1.2 (10)	3.4 $\pm$ 0.3 <sup>‡</sup> (10)	63

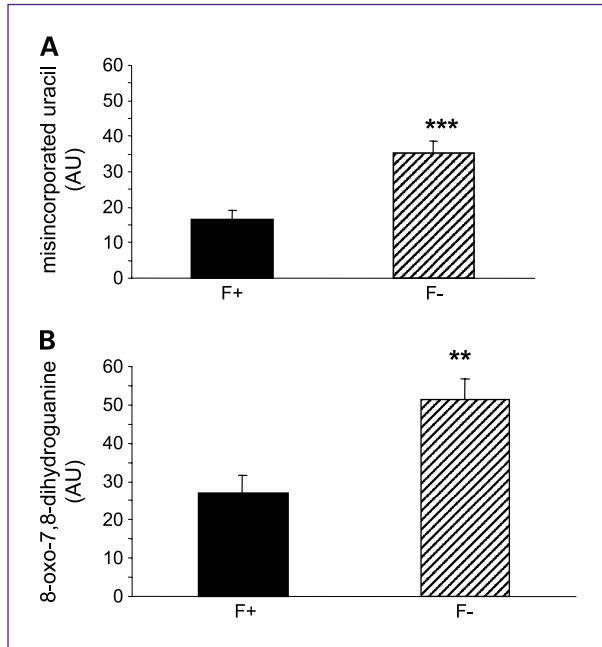
NOTE: Values are the mean  $\pm$  SE, with number of rats sampled per group in parentheses. Significance refers to differences between F<sup>+</sup> versus F<sup>-</sup> animals at the end of the experiment.

\* $P < 0.05$ .

<sup>†</sup> $P < 0.01$ .

<sup>‡</sup> $P < 0.001$ .

<sup>§</sup> $P < 0.0001$ .



**Fig. 3.** The effect of folic acid deficiency on misincorporated uracil (A) and 8-oxo-7,8-dihydroguanine (B) levels in rat lymphocytes. Rats were fed either a folate-sufficient diet (F<sup>+</sup>; black columns) or a diet deficient in folic acid (F<sup>-</sup>; hatched columns) for 24 wk. Columns, mean values (in arbitrary units;  $n = 8$  per group); bars, SE. \*\*,  $P < 0.003$ ; \*\*\*,  $P < 0.001$ , where significance refers to differences between F<sup>+</sup>- and F<sup>-</sup>-treated rats at each time point.

(~11%;  $P < 0.004$ ; Table 3). Folate deficiency caused a similar drop in SAM:SAH in the distal colon (Table 2). DNA methyltransferase activity was elevated in folate-deficient rat liver (Table 3). DNA was hypomethylated in the liver

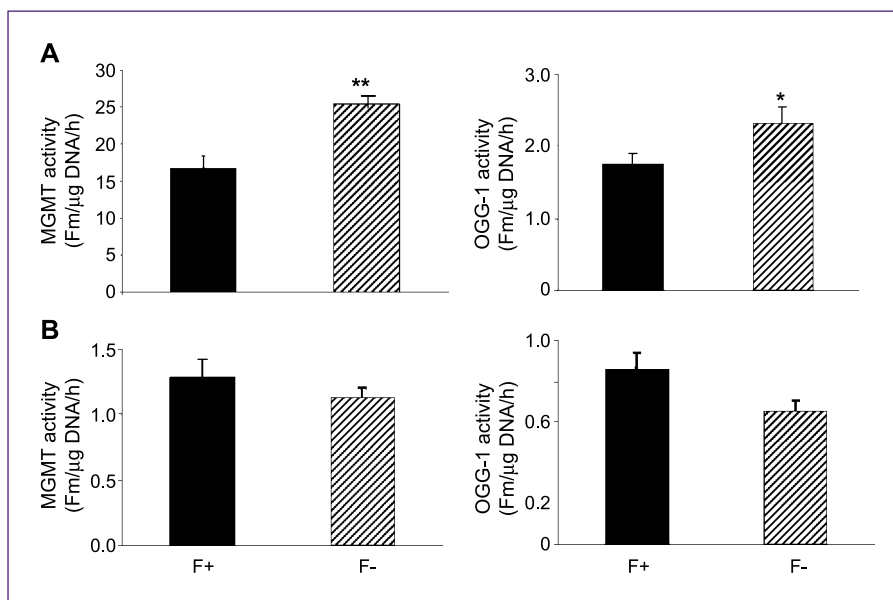
and colon from folate-deficient animals compared with controls (Table 3). 5-Methyldeoxycytidine concentrations and percentage methylated DNA (measured by LC MS/MS) were both lower in these tissues. However, none of these differences reached statistical significance.

## Discussion

Folate is essential for the biosynthesis, repair, and methylation of DNA. High folate status (based on measures of dietary intake and blood levels) is generally associated with a decreased risk of certain human cancers, including colorectal cancer (1, 2). However, there is real concern about a potential harmful role for long-term intervention with high doses of synthetic folic acid. Findings from recent human observational and placebo-controlled intervention trials, or analyses of cancer incidence data, suggest that supplementation with synthetic folic acid may promote progression of initiated cancer cells at several sites, including the breast, colon, and prostate (6–9).

Nonetheless, evidence from the majority of studies indicates that habitual consumption of natural folates from the diet reduces cancer development, whereas folate deficiency increases risk (1, 2, 10). Folate deficiency perturbs nucleotide synthesis, increases DNA damage, and alters global and gene-specific methylation. The effect that folate deficiency has on DNA repair *in vivo* has not been extensively examined.

Defective DNA repair is linked to human cancer development (17, 18). Mutations in mismatch repair genes are associated with heritable colon cancer (17), whereas polymorphisms in specific repair genes (e.g., *hOGG1*, *XRCC1*, and *PARP1*) correlate with altered cancer risk and progression of colorectal cancer (20, 32). Folate is essential for the



**Fig. 4.** The effect of folic acid deficiency on MGMT and OGG-1 DNA repair activity in rat liver (A) and colon (B). Rats were fed either a folate-sufficient diet (F<sup>+</sup>; black columns) or a diet deficient in folic acid (F<sup>-</sup>; hatched columns) for 24 wk. Columns, mean ( $n = 12$  per group); bars, SE. \*,  $P < 0.03$ ; \*\*,  $P < 0.003$ , where significance refers to differences between F<sup>+</sup>- and F<sup>-</sup>-treated rats at each time point.

**Table 3.** Indices of methyl donor status and global DNA methylation in blood and tissues from rats fed a folate-supplemented (F<sup>+</sup>) or folate-deficient (F<sup>-</sup>) diet for 24 wk

	Methylation status	
	F <sup>+</sup>	F <sup>-</sup>
Plasma total homocysteine (μmol/L)	5.01 ± 0.27 (12)	6.91 ± 0.32* (12)
Plasma B12 (pg/mL)	503.1 ± 40.5 (10)	579.3 ± 53.5 (10)
Lymphocyte B12 (pg/10 <sup>5</sup> cells)	13.94 ± 0.40 (10)	12.39 ± 0.70 (10)
Liver B12 (pg/mg protein)	366.8 ± 27.1 (10)	437.8 ± 44.7 (10)
Distal colon B12 (pg/mg protein)	622.7 ± 50.5 (10)	618.2 ± 78.6 (10)
Liver SAM	81.60 ± 4.93 (10)	79.22 ± 4.99 (10)
Liver SAH	14.53 ± 0.9 (10)	15.78 ± 1.06 (10)
Liver SAM:SAH ratio	5.63 ± 0.12 (10)	5.04 ± 0.13 <sup>†</sup> (10)
Colon SAM	32.47 ± 4.36 (10)	28.36 ± 2.91 (10)
Colon SAH	3.57 ± 0.47 (10)	4.48 ± 0.52 (10)
Colon SAM:SAH ratio	9.75 ± 1.34 (10)	7.09 ± 1.03 (10)
Liver DNA methyltransferase (absorbance/h/mg nuclear protein)	4.38 ± 0.90 (12)	6.70 ± 2.33 (12)
Liver 5-methyldeoxycytidine (ng/μg DNA; LC MS/MS)	2.62 ± 0.32 (12)	2.12 ± 0.23 (12)
Liver genomic DNA methylation (%; LC MS/MS)	4.63 ± 0.10 (12)	4.54 ± 0.04 (12)
Colon 5-methyldeoxycytidine (ng/μg DNA; LC MS/MS)	4.56 ± 0.22 (12)	3.47 ± 0.51 (12)
Colon genomic DNA methylation (%; LC MS/MS)	4.87 ± 0.09 (12)	4.61 ± 0.15 (12)

NOTE: Values are the mean ± SE, with number of rats sampled per group in parentheses. Significance refers to differences between F<sup>+</sup> versus F<sup>-</sup> animals at the end of the experiment.

\*P = 0.02.

<sup>†</sup>P = 0.004.

repair of DNA. In the present study, we investigated whether a moderate folate deficiency in rats altered the repair capacity for two lesions implicated in human cancer etiology. The strongly mutagenic oxidized base 8-oxo-7,8-dihydroguanine occurs in significant quantities in human DNA and induces G:C to T:A transitions (21). Removal of this lesion by base excision repair (BER) is achieved by OGG-1. O<sup>6</sup>-Methylguanine is detectable in human colonic DNA (22) and at high levels in tumor-prone regions of the human bowel (23). It is potentially cytotoxic, mutagenic (causing G:C to A:T transitions and recombinations), and carcinogenic unless repaired by MGMT. Decreased activity of MGMT in normal human colorectal tissue and cancerous tissue is strongly associated with G:C to A:T mutations in the K-ras proto-oncogene (23).

We firstly showed that feeding rats a folate-deficient diet for 24 weeks caused moderate intracellular folate deficiency. Blood cell folate dropped by 40% and tissue folate by 25% to 60%. Liver folate was depleted ~30% and colon 60%. These data are consistent with a study carried out by us in rats fed the same diet for 8 weeks, where whole blood and liver folate decreased 29% and 26%, respectively (25). Similar folate levels have been reported in rats fed a folate-free diet (without succinyl sulfathiazole) for 15 weeks. Here, serum folate was reduced by 54%, liver folate by 67%, and colon folate by 30%.

This was described as a mild folate deficiency (24). Conversely, feeding rodents succinyl sulfathiazole, together with a folate-free diet, induces a much more severe deficiency. Plasma folate was depleted 93% over 8 weeks in DNA repair-deficient mice fed this diet (26) and liver folate by 95% in normal mice after only 4 weeks (27). In addition to the effects of the deficient diet on folate levels, we also observed a 2-fold increase in uracil misincorporation. In this study, uracil misincorporation served as a sensitive intracellular marker of folate status and function. We have shown previously that uracil misincorporation *in vitro* and in rats is increased in a concentration- or time-dependent manner (25, 33, 34). Uracil is also present at detectable levels in human DNA (31) and is increased by folate depletion (15) and reduced by supplementation (35).

We have reported recently that DNA repair enzymes are significantly upregulated in folate-depleted cells *in vitro* (33). Proteomic analysis of human NCM460 cells grown in folate-deficient medium showed significant changes in expression of DNA repair enzymes, including MSH2 and XRCC5, involved in mismatch repair and double-strand break repair, respectively. Despite this upregulation in repair enzyme activity, BER of oxidation and alkylation damage is compromised in folate-deficient human lymphocytes, in normal human colon epithelial cells, and in rat colon cells *ex vivo* (24, 33, 34). Increased repair enzyme

expression seems counterintuitive given the evidence that folate deficiency profoundly inhibits repair *in vitro* (24, 33, 34). However, single-cell gel electrophoresis, the assay used in these *in vitro* experiments, assesses repair completed by the full complement of enzymes contained within the cell and not only the initial excision steps in the repair process. It may be that whereas folate deficiency upregulates the activity of initial DNA incision repair enzymes, other enzymes (e.g., DNA polymerases downstream in the repair process) remain unchanged and thus complete repair is compromised. Damaged bases are removed by DNA glycosylases in BER, and upregulation of certain of these repair enzymes has been reported in repair-deficient mice, without a corresponding increase in  $\beta$ -polymerase activity (26). Increased incision and excision without subsequent patch repair would increase DNA strand breakage. This, together with depletion of the nucleotide precursor pool and/or changes in the balance of DNA precursors as a consequence of folate deficiency (36, 37), would result in the accumulation of DNA strand breaks as observed by Cabelof et al. (26) and detected as DNA repair inhibition in the single-cell gel electrophoresis assay (24, 33, 34).

An important and novel finding of this study was that folate deficiency significantly increased hepatic OGG-1 and MGMT repair activity (~25% for both proteins). Exactly how folate deficiency modulates DNA repair remains to be established. BER (26) and particularly rat MGMT are DNA damage inducible (38), and a wide range of genotoxic agents is able to elicit this response most extensively in the liver (39). From such observations, it is reasonable to suggest that upregulation of these two proteins indicates the occurrence of DNA damage, lending further support to the finding that increased DNA damage (including DNA strand breaks, uracil misincorporation, and oxidized bases) is a consequence of folate deficiency (15, 25, 34). Although we attempted to quantify the levels of *O*<sup>6</sup>-methylguanine and 8-oxo-7,8-dihydroguanine directly in rat liver and colon DNA, they were below the limit of detection for the assays used (data not shown). We cannot therefore report whether MGMT and OGG-1 activity was elevated by increased concentrations of substrate lesions specifically in these tissues. However, folate deficiency significantly increased 8-oxo-7,8-dihydroguanine levels in DNA in lymphocytes from rats fed the folate-deficient diet.

Although there were highly significant changes in OGG-1 and MGMT expression in the rat liver in response to folate depletion, no such effects were seen in the colon, indicating that the ability of the liver to respond to folate deficiency is not shared by the colon. Given the evidence that hepatic upregulation is triggered by DNA damage, it is reasonable to speculate that the colon cannot respond as robustly as the liver to such damage and would therefore be more susceptible to the genotoxic effects instigated by folate deficiency. An alternative explanation, that colon does not suffer the same extent of DNA damage as the liver, seems less likely.

A key question is whether the findings from this animal study can be extrapolated to humans. Little is known currently about the effect of folate status on DNA repair in humans. Nucleotide excision repair is impaired in lymphocytes from individuals with poor folate status (40). Conversely, supplementing healthy volunteers of adequate folate status with folic acid (1.2 mg/d for 12 weeks) does not alter BER-mediated excision of 8-oxo-7,8-dihydroguanine from lymphocytes (35). Although these data from surrogate tissues suggest that DNA repair activity is suboptimal in people with low folate intake and may not be improved in individuals with satisfactory folate status, a decrease in BER after intervention was observed in subjects with the lowest baseline red cell folate (35), suggesting that DNA repair may be sensitive to both positive and negative changes in folate status. Whether the colon is less refractive to folate deficiency in people and whether this has a negative effect on genomic stability and malignant transformation remain to be discovered. We have shown previously that MGMT activity in normal human colorectal mucosa is inversely associated with vegetable consumption and that high dietary folate intake is related to low DNA alkylation damage (N7-methylguanine; ref. 41). Similarly, markers of microsatellite instability are decreased in patients with ulcerative colitis (a condition predisposing to colorectal cancer) treated with very high doses of folic acid (5 mg/d for 6 months; ref. 13).

To complement the repair aspect of this study, we determined the effect of folate deficiency on key intermediates in the methionine cycle (tissue SAM and SAH, plasma homocysteine, plasma, lymphocyte, and tissue B12) and genome-wide DNA methylation, a common feature in tumorigenesis (reviewed in refs. 2, 14). Liver and colon DNA was hypomethylated approximately 2% and 5%, respectively, in rats fed a folate-deficient diet relative to DNA from animals in the control group. However, despite a progressive reduction in blood and tissue folate concentrations, an increase in plasma homocysteine, and a decrease in the liver SAM to SAH ratio, folate deficiency did not statistically significantly alter genome-wide DNA methylation in these tissues.

The reported effect of folate deficiency on DNA methylation is highly variable and profoundly dependent on the treatment regimen, tissue, and genes examined (reviewed in ref. 2). Generally, severe folate deficiency *in vitro*, in rodents, and in humans causes DNA hypomethylation, whereas moderate deficiency is ineffective (reviewed in refs. 2, 33, 42–44). Our data do not support the hypothesis that folate deficiency induces measurable DNA hypomethylation *in vivo*, suggesting that epigenetic changes are not relevant to any biological effect of folate modulation in our rat model.

In conclusion, moderate but prolonged folate deficiency significantly altered DNA repair activity in rat liver but not in colon. This may reflect the ability of the liver, but not the colon, to upregulate DNA repair enzymes

in response to elevated DNA damage. If this inability of colon tissue to upregulate DNA repair processes occurs in humans, it may constitute one of the mechanism through which folate deficiency increases the potential for malignant transformation.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### References

- Giovannucci E. Epidemiologic studies of folate and colorectal neoplasia: a review. *J Nutr* 2002;132:2350–5S.
- Kim YI. Folate and colorectal cancer: an evidence-based critical review. *Mol Nutr Food Res* 2007;51:267–92.
- Kim YI, Salomon RN, Graeme-Cook F, Choi SW. Dietary folate protects against the development of macroscopic colonic neoplasia in a dose-dependent manner in rats. *Gut* 1996;39:732–40.
- Song J, Medline A, Mason JB, Gallinger S, Kim Y-I. Effects of dietary folate on intestinal tumorigenesis in the Apc<sup>Min</sup> Msh mouse. *Cancer Res* 2000;60:5434–40.
- Song J, Sohn K-J, Medline A, Ash C, Gallinger S, Kim Y-I. Chemopreventive effects of dietary folate on intestinal polyps in Apc<sup>+/–</sup> Msh<sup>–/–</sup> mice. *Cancer Res* 2000;60:3191–9.
- Charles D, Ness AR, Campbell D, Smith GD, Hall MH. Taking folate in pregnancy and risk of maternal breast cancer. *BMJ* 2004;329:1375–6.
- Cole BF, Baron JA, Sandler RS, et al. Folic acid for the prevention of colorectal adenomas—a randomized clinical trial. *JAMA* 2007;297:2351–9.
- Mason JB, Dickstein A, Jacques PF, et al. A temporal association between folic acid fortification and an increase in colorectal cancer rates may be illuminating important biological principles: a hypothesis. *Cancer Epidemiol Biomarkers Prev* 2007;16:1325–9.
- Figueiredo JC, Grau MV, Haile RW, et al. Folic acid and risk of prostate cancer: results from a randomised clinical trial. *J Natl Cancer Inst* 2009;101:432–5.
- Sanjoaquin MA, Allen N, Couto E, Roddam AW, Key TJ. Folate intake and colorectal cancer risk: a meta-analytical approach. *Int J Cancer* 2005;113:825.
- Scientific Advisory Committee on Nutrition. Folate and disease prevention. London: The Stationery Office; 2006.
- De Benoist B, Allen LH, Rosenberg IH. Folate and vitamin B12 deficiencies: proceedings of a WHO technical consultation. *Food Nutr Bull* 2008;29:S1–246.
- Cravo ML, Albuquerque CM, de Sousa S, et al. Microsatellite instability in non-neoplastic mucosa of patients with ulcerative colitis: effect of folate supplementation. *Am J Gastroenterol* 1998;93:2060–4.
- Arasaradam RP, Commane DM, Bradburn D, Mathers JC. A review of dietary factors and its influence on DNA methylation in colorectal carcinogenesis. *Epigenetics* 2008;3:193–8.
- Blount BC, Mack MM, Wehr WM. Folate deficiency causes uracil misincorporation into human DNA and chromosomal breakage: implications for cancer and neuronal damage. *Proc Natl Acad Sci U S A* 1997;94:3290–5.
- Berger SH, Pittman DL, Wyatt MD. Uracil in DNA: consequences for carcinogenesis and chemotherapy. *Biochem Pharmacol* 2008;76:697–706.
- Whitehouse A, Meredith DM, Markham AF. DNA mismatch repair genes and their association with colorectal cancer (review). *Int J Mol Med* 1998;1:469–74.
- De Boer J, Hoeijmakers JHJ. Nucleotide excision repair and human syndromes. *Carcinogenesis* 2000;21:453–60.
- Skjelbred CF, Svendsen M, Haugan V, et al. Influence of DNA repair gene polymorphisms of hOGG1, XRCC1, XRCC3, ERCC2 and the folate metabolism gene MTHFR on chromosomal aberration frequencies. *Mutat Res* 2006;602:151–62.
- Goode EL, Ulrich CM, Potter JD. Polymorphisms in DNA repair genes and associations with cancer risk. *Cancer Epidemiol Biomarkers Prev* 2002;11:1513–30.
- Fortini P, Pascucci B, Parlanti E, D'Errico M, Simonelli V, Dogliotti E. 8-Oxoguanine DNA damage: at the crossroads of alternative repair pathways. *Mutat Res* 2003;531:127–39.
- Jackson RE, Hall CN, Badawi AF, O'Conner PJ, Cooper DP, Povey AC. The frequency of K-ras mutations and DNA alkylation in colorectal tissue from individuals living in Manchester. *Mol Carcinogen* 1996;16:12–9.
- Povey AC, Lees NP, Harrison KL, Hall CN, Margison GP. Altered O<sup>6</sup>-alkylguanine DNA alkyltransferase (MGMT) activity in normal colorectal tissue and adenomas is associated with K-ras GC-AT gene mutations. *Proc Am Assoc Cancer Res* 2002;5049:1020.
- Choi S-W, Kim Y-I, Weitzel JN, Mason JB. Folate depletion impairs DNA excision repair in the colon of the rat. *Gut* 1998;43:93–9.
- Duthie SJ, Grant G, Narayanan S. Increased uracil misincorporation in lymphocytes from folate-deficient rats. *Brit J Cancer* 2000;83:1532–7.
- Cabelof DC, Raffoul JJ, Nakanura J, Kapoor D, Abdalla H, Heydari AR. Imbalanced base excision repair in response to folate deficiency is accelerated by polymerase  $\beta$  haploinsufficiency. *J Biol Chem* 2004;279:36504–13.
- Branda RF, O'Neill JP, Brooks EM, Powden C, Naud SJ, Nicklas JA. The effect of dietary folic acid deficiency on the cytotoxic and mutagenic responses to methyl methanesulfonate in wild-type and in 3-methyladenine DNA glycosylase-deficient Aag null mice. *Mutat Res* 2007;615:12–7.
- Watson AJ, Margison GP. O<sup>6</sup>-alkylguanine-DNA alkyltransferase assay. *Methods Mol Biol* 2000;152:49–61.
- Watson AJ, Margison GP. Assays for the repair of oxidative damage by formamidopyrimidine glycosylase (Fpg) and 8-oxoguanine DNA glycosylase (OGG-1). *Methods Mol Biol* 2000;152:17–32.
- Wang W, Kramer PM, Yang S, Pereira MA, Tao L. Reversed-phase high performance liquid chromatography procedure for the simultaneous determination of S-adenosyl-L-methionine and S-adenosyl-L-homocysteine in mouse liver and the effect of methionine on their concentrations. *J Chromatogr B* 2001;762:59–65.
- Friso S, Choi SW, Girelli D. A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. *Proc Natl Acad Sci U S A* 2002;99:5606–11.
- Bigler J, Ulrich CM, Kawashima T, Whitton J, Potter JD. Polymorphisms in hOGG1, XRCC1 and XRCC3 as risk factors for colorectal polyps. *Proc Am Assoc Cancer Res* 2006;47:2051.
- Duthie SJ, Mavrommatis Y, Rucklidge G, et al. The response of human colonocytes to folate deficiency *in vitro*: functional and proteomic analysis. *J Proteome Res* 2008;7:3254–66.
- Duthie SJ, Narayanan S, Blum S, Pirie L, Brand GM. Folate deficiency *in vitro* induces uracil misincorporation, DNA hypomethylation and inhibits DNA excision repair in immortalised normal human colon epithelial cells. *Nutr Cancer* 2000;37:127–33.
- Basten GP, Duthie SJ, Pirie L, Vaughan N, Hill MH, Powers HJ. Sensitivity of markers of DNA stability and DNA repair activity



- to folate supplementation in healthy volunteers. *Brit J Cancer* 2006; 94:1942–7.
36. James SJ, Yin L. Diet induced DNA damage and altered nucleotide metabolism in lymphocytes from methyl-donor-deficient rats. *Carcinogenesis* 1998;10:1209–14.
  37. James SJ, Pogribny IP, Pogribna M, Miller BJ, Jernigan S, Melnyk S. Mechanisms of DNA damage, DNA hypomethylation and tumour progression in the folate/methyl deficient rat model. *J Nutr* 2003; 133:3740–47S.
  38. O'Connor PJ, Margison GP. The enhanced repair of O<sup>6</sup>-alkylguanine in mammalian systems. In: Seeberg E, Kleppe K, editors. *Chromosome damage and repair*. London: Plenum Press; 1981, p. 233–45, Proceedings of the NATO-EMBO Advanced Study Institute Series A, Life Sciences 40.
  39. O'Connor PJ, Chu Y-H, Cooper DP, Maru GB, Smith RA, Margison GP. Species differences in the inducibility of hepatic O<sup>6</sup>-alkylguanine repair rodents. *Biochimie* 1982;64:769–73.
  40. Wei Q, Shen H, Wang LE. Association between low dietary folate intake and suboptimal cellular DNA repair capacity. *Cancer Epidemiol Biomarker Prev* 2003;12:963–9.
  41. Billson HA, Harrison KL, Lees NP, Hall CN, Margison GP, Povey AC. Dietary variables associated with DNA N7-methylguanine and O<sup>6</sup>-alkylguanine DNA-alkyltransferase activity in human colorectal mucosa. *Carcinogenesis* 2009;30:615–20.
  42. Wasson GR, McGlynn AP, McNulty H, et al. Global DNA and p53 region-specific hypomethylation in human colonic cells is induced by folate depletion and reversed by folate supplementation. *J Nutr* 2006;136:2748–53.
  43. Ingrosso D, Cimmino A, Perna AF, Masella L. Folate treatment and unbalanced methylation and changes of allelic expression induced by hyperhomocystenemia in patients with uremia. *Blood* 2003;361:1693–9.
  44. Fenech M, Aitken C, Rinaldi J. Folate, vitamin B12, homocysteine status and DNA damage in young Australian adults. *Carcinogenesis* 1998;7:1163–71.