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Multiple B-vitamin inadequacy amplifies alterations induced by folate depletion in *p53* expression and its downstream effector *MDM2*

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Abstract

Folate is required for biological methylation and nucleotide synthesis, aberrations of which are thought to be the mechanisms that enhance colorectal carcinogenesis produced by folate inadequacy. These functions of folate also depend on the availability of other B-vitamins that participate in “one-carbon metabolism,” including B2, B6 and B12. Our study therefore investigated whether combined dietary restriction of these vitamins amplifies aberrations in the epigenetic and genetic integrity of the *p53* gene that is induced by folate depletion alone. Ninety-six mice were group pair-fed diets with different combinations of B-vitamin depletion over 10 weeks. DNA and RNA were extracted from epithelial cells isolated from the colon. Within the hypermutable region of *p53* (exons 5–8), DNA strand breaks were induced within exons 6 and 8 by folate combined with B2, B6 and B12 restriction ($p < 0.05$); such effects were not significantly induced by mild folate depletion alone. Similarly, a minor degree of hypomethylation of exon 6 produced by isolated folate depletion was significantly amplified ($p \leq 0.05$) by simultaneous depletion of all 4 B-vitamins. Furthermore, the expression of *p53* and *MDM2* were significantly decreased ($p \leq 0.05$) by the combined depletion state but not by folate depletion alone. These data indicate that inadequacies of other 1-carbon vitamins may amplify aberrations of the *p53* gene induced by folate depletion alone, implying that concurrent inadequacies in several of these vitamins may have added tumorigenic potential beyond that observed with isolated folate depletion.

Keywords

B-vitamin; methylation; *p53*; strand breaks

The water-soluble B vitamin, folate, is an essential cofactor that provides 1-carbon moieties for DNA methylation and other epigenetic processes as well as for the biosyntheses of purine

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and thymidine nucleotides.¹ Aberrations in these processes are thought to play important roles in carcinogenesis and are thought to be the avenues through which folate inadequacy enhances carcinogenesis.^{2–4}

However, maintaining the integrity of biological methylation and nucleotide synthesis depends not only upon the adequate availability of folate but also on the adequate availability of other “1-carbon nutrients,” including vitamins B2, B6 and B12 (Fig. 1). Methylene tetrahydrofolate reductase (MTHFR), which catalyzes the conversion of 5,10-methylene tetrahydrofolate to 5-methyl tetrahydrofolate, is a flavoprotein.⁵ As a result, modest decreases in dietary riboflavin (B2) intake in human populations in concert with the presence of a common polymorphism in the MTHFR gene collectively impair folate-dependent methionine synthesis, resulting in a phenotype of hyperhomocysteinemia.⁷ Similarly, it is known that vitamin B12 deficiency produces a functional folate deficiency by producing a “methylfolate trap” and it has been shown that B12 deficiency alone can produce abnormalities in both DNA methylation and uracil incorporation,⁸ thereby mimicking some of the effects of folate depletion. Moreover, vitamin B6 is a necessary cofactor for the interconversion of other coenzymatic forms of folate,⁹ and is a cofactor for cystathionine β -synthase as well, mediating the trans-sulfuration of homocysteine to cystathionine.⁶ Therefore it is also intimately linked, in a metabolic sense, to the cellular functions of folate. Thus, the metabolic functions of all these 1-carbon vitamins are highly interdependent such that depletion of one often leads to biochemical phenotypes characteristic of deficiencies of the others.

It is perhaps not surprising, therefore, that in addition to the compelling body of literature linking low folate status with an increased risk of colorectal cancer^{10–13} are studies that have begun to associate decreased vitamin B2, B6 and B12 status with colorectal neoplasms as well. In a case-control study, habitual vitamin B2 intake was inversely associated with colorectal adenomas.¹⁴ Similarly, vitamin B6 status, measured either by habitual intake¹⁵ or blood levels,¹⁶ has been observed to be protective against colorectal adenomas and cancers in 2 large prospective cohort studies. An animal study also provided corroborative evidence for a protective effect of B6.¹⁷ Vitamin B12 intake, when present in conjunction with higher folate intake, has also been linked to a lower risk of colorectal cancer in a prospective cohort study,¹⁶ and this joint effect with folate is consistent with a study in a rodent model in which B12 depletion replicated the effects of folate depletion on DNA methylation and uracil incorporation within the colonic mucosa.⁸

Impaired or abnormal expression of the *p53* tumor suppressor gene are among the most frequent and fundamentally important molecular abnormalities that occur in colorectal carcinogenesis.¹⁹ It is of considerable interest that experimental folate inadequacy has been shown to induce both hypomethylation and DNA strand breaks within the coding region of the gene.^{20–22} A potentially important functional consequence of the anomalies in methylation and strand integrity produced by folate inadequacy is impaired expression of the gene. Consistent with this observation is the observation that depletion of a severe degree in the intact animal suppresses colonic *p53* expression in conjunction with the induction of strand breaks.²¹

Severe folate deficiency is uncommon in industrialized nations but mild inadequacies of multiple B-vitamins occur frequently, and commonly in combination. Population-based studies have reported that 18–25% of adolescents and adults have blood indicators of B6 status that fall below the accepted lower limits of normality in the United States and Western Europe.^{23,24} Also, up to 15–20% of healthy elders are thought to have marginal B12 status.²⁵ Therefore, to model more closely what occurs in industrialized societies, we chose to examine the effects of very mild states of multiple B vitamin depletion. We hypothesized that aberrant biochemical and molecular pathways in the colon due to impaired 1-carbon metabolism are amplified in the presence of inadequacies of multiple 1-carbon vitamins in a manner that would not

otherwise be observed with isolated folate depletion. In our study, we examined select features of the *p53* tumor suppressor gene in animals with no underlying predisposition to cancer since we wished to explore how potential synergies of multiple deficiency states might produce a procarcinogenic milieu in the absence of a genetic drive toward or carcinogen-induced colon carcinogenesis.

Material and methods

Animals and diets

The animal protocol was approved by the Institutional Animal Care and Use Committee of the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University. C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed individually in wire-bottomed stainless steel cages in a temperature controlled (24°C) room, given *ad libitum* access to water. Group paired-feeding was used to minimize the effect from body weight. Mice were weighed weekly and weight increased progressively during the 10-week experiment, but there were no differences among these groups. All mice survived the entire experimental period.

Ninety-six 4-month-old mice were weight-matched and randomly assigned to 1 of 6 amino acid-defined diets,²⁶ each containing different levels of folic acid, vitamins B2, B6 and B12 (Table I). The diet was formulated precisely as originally described by Walzem and Clifford²⁶ except that pectin was substituted for cellulose as the source of fiber, since the former adsorbs dietary B12 and thereby facilitates its depletion. This diet has a moderately increased content of methionine (~2.5-fold) compared to the AIN-93 diet.²⁷ Although this could theoretically mask the development of related one-carbon nutrients, the diet has been used extensively to induce various degrees of folate depletion,^{28,29} as well as a secondary depletion of choline that accompanies folate depletion.³⁰ All diets were made by Harlan Teklad (Indianapolis, IN), and the dietary vitamin concentrations were chemically analyzed and confirmed by Covance (Madison, WI; data not shown). We also confirmed the folic acid content of each diet with the microbiological assay³¹ and found that the actual concentrations never varied more than 10% from the stated value (data not shown). Although no folic acid was provided in the folate-deplete diet, succinylsulfathiazole was not added and therefore folate synthesis by the intestinal microflora was able to occur, which prevents the development of severe folate deficiency.²⁶

Blood and tissue collection

After 10 weeks of feeding, all mice were killed. Mice were anesthetized with isoflurane and exsanguinated by cardiac puncture. Colonocytes were isolated from 8 mice in each group with 30 mM EDTA at 4°C, as previously described,³² for subsequent DNA and RNA extraction for measurements of region-specific DNA methylation, strand breaks and gene expression. The colons from each of the other 8 mice in each group was placed atop a glass plate at 4°C and gently scraped with the edge of a glass slide in order to isolate the epithelium; these scrapings were subsequently used for the tissue folate assay. Both mucosal scrapings and isolated colonocytes were stored at -70°C until they were used for the indicated analyses.

Measurement of blood and tissue vitamin status

Plasma folate concentrations were determined by radioimmunoassay (Bio-Rad Laboratories, Hercules, CA), whereas colonic mucosal folate was measured by a standard microbiologic microtiter plate assay using *Lactobacillus casei* after folate extraction and subsequent treatment with chicken pancreas conjugase, as previously described.^{33,34} Vitamin B2 was measured by erythrocyte glutathione reductase activity assay.³⁵ Vitamin B6 was determined by

radioenzymatic assay³⁶; vitamin B12 was measured by competitive protein binding assay (Bio-Rad Laboratories).

Determination of strand breaks within the *p53* hypermutable region

Exons 5–8 of the *p53* gene were chosen because previous animal studies have shown that this region is more susceptible to strand breakage and hypomethylation due to dietary folate depletion than other, less highly conserved exonic regions of the gene^{20,37} and because most *p53* mutations in human cancer occur in this region. The detection of *p53* exon-specific strand breaks was determined by a PCR method previously used by both our lab and others,³⁸ which is based on the principle that preexisting lesions in DNA halts the progression of *Taq* polymerase during PCR amplification. A novel primer set for each exon was designed (Table II) and its ability to quantitatively assess its target region was demonstrated by examining the effect of decreasing amounts of template on the increment of amplification (Ct value) (Fig. 2a). A 46-bp segment of β -actin was amplified as a control region for the real-time PCR reaction, and the amplification of this segment was verified to be not significantly changed among the different dietary groups. *p53* exon 5–8 breaks are reported as a Δ Ct value ($Ct_{p53\text{exon}5-8} - Ct_{\beta\text{-actin}}$), with a higher Δ Ct indicating a lower template integrity.

Quantification of DNA methylation on *HpaII* cleavage site at *p53* hypermutable region

Because the *HpaII* restriction enzyme is blocked by cytosine methylation, the methylation status of its recognition site (CCGG) can be assessed with quantitative PCR by using primers flanking the *HpaII* cutting sites of interest.³⁹ If the cytosine on the recognition site (CCGG) is methylated, *HpaII* can not cut it and the DNA template remains intact. Conversely, if the cytosine on the recognition site (CCGG) is unmethylated, *HpaII* will cut it and the DNA template is broken. Therefore, higher degrees of methylation level at the recognition site (CCGG) result in more intact template and more PCR product. There are 2 *HpaII* cleavage sites on exons 5–8, the hypermutable region, with 1 CCGG site on exon 6 which has 5 CpG sites and the other on exon 7 which has 3 CpG sites (Fig. 3a, NCBI Gene ID: 22059).

Colonic DNA sample was digested with *HpaII* (New England Biolabs, Ipswich, MA) at a final concentration of 2 U/ μ g DNA at 37°C for 16 h. After digestion, the incubation mixture was heated at 65°C for 20 min to inactivate *HpaII* before PCR amplification. The amplification of exon 6 and 7 was performed on a 7300 realtime PCR machine in a 20 μ l reaction containing SYBR Green PCR master mix (Applied Biosystems, Foster City, CA), 750 nM of each primer and 50 ng digested DNA. The same amount of undigested DNA was amplified to serve as a control. The same primers used in the above strand break assays for exons 6 and 7 were used here, since those primer sets were validated by testing the effect of decreasing amounts of template on the increment of amplification (Ct value). Thermal cycling was performed as follows: 95°C, 10 min, followed by 40 cycles of 95°C, 15 sec, and 60°C, 75 sec and finally a dissociation profile. The methylation status of the *HpaII* cutting sites on *p53* exons 6 and 7 are reported as a Δ Ct value ($Ct_{\text{digested DNA}} - Ct_{\text{undigested DNA}}$), with a higher Δ Ct indicating a lower methylation.

Real-time PCR assay for *p53*, *MDM2* and *p21* mRNA expression

Total RNA was isolated from colonic epithelium with Trizol reagent (Invitrogen, Carlsbad, CA), and the first strand cDNAs were synthesized using Oligo d(T)_{xxx} and Superscript II reverse transcriptase (Invitrogen). The expression of the *p53* and *MDM2* genes was quantified using Taqman Gene Expression Assays, and an ABI7300 real-time PCR machine (Applied Biosystems). Gene expression was normalized to β -actin (Δ Ct = $Ct_{\text{target genes}} - Ct_{\beta\text{-actin}}$), and statistical analyses were performed using Δ Ct. The relative expression values compared to the folate sufficient group are reported here for clarity; and were calculated using the following formula: relative expression = $2^{-\Delta\Delta\text{Ct}}$, where $\Delta\Delta$ Ct is (Δ Ct of vitamin depletion groups) –

(ΔC_t of folate sufficient group). For *p53*, the cDNA was equally pooled from each mouse for each group and a regular RT-PCR was performed with cycles within the linear amplification range to confirm the real-time result.

Statistical analysis

Data analyses were conducted by one-way ANOVA for the dietary effect, with comparisons being made by the Fisher LSD method. Statistical analysis was performed with SAS software v9.1 (SAS Institute, Cary, NC). Values are presented as mean $\pm$ SEM.

Results

Vitamin status in blood and tissue

A 35–55% reduction of plasma folate was induced ($p \leq 0.05$) by mild dietary folate depletion compared to the folate sufficient group (Table III). Consistent with the plasma folate, folate depletion also induced a reduction in colonic folate concentrations that were ~30–55% lower in folate deplete groups (Table III). A significant correlation ($R = 0.51$, $p < 0.01$) existed between plasma and colonic folate concentrations based on a Pearson correlation analysis.

Plasma vitamin B6 (pyridoxal 5'-phosphate) and B12 were each about 50% lower ($p \leq 0.05$) in the deplete group than in groups replete of these vitamins. Erythrocyte vitamin B2, which was measured in a reciprocal fashion by the activation coefficient, was also lower ($p < 0.05$) in the groups exposed to depletion of B2 compared to the folate sufficient group. Plasma homocysteine, a sensitive indicator of folate status, increased 40.7% in the multiple vitamin depletion group compared to the folate sufficient group (Table III).

These data on vitamin status also appear in a separate publication⁴⁰ in which tissues and blood from this experiment were used to examine how vitamin depletion alters components of the Wnt pathway. The remaining data reported here have not been presented elsewhere.

Strand breaks in the *p53* hypermutable region

Four primer sets were designed for *p53* exons 5–8, the mutation hot-spot region. PCR amplification efficiencies of these 4 primer sets were checked with increasing amounts of template DNA concentration in the reaction mixture from 0.625 to 10 ng/ μ l. A linear increase of reaction cycle threshold (C_t) was observed with decreasing the template DNA concentration (Fig. 2a).

Using the above 4 primer sets, strand breaks within exons 5–8 of *p53* were determined by real-time PCR. No significant induction of strand breaks ($p > 0.05$) was observed in exons 5 and 7 in response to single, double or multiple B-vitamin depletion (data not shown). For exons 6 and 8, single and double vitamin depletion did not significantly alter strand fidelity, while multiple depletions induced a significant elevation in breaks (Fig. 2b, $p < 0.05$).

DNA methylation on *HpaII* cleavage site in the *p53* hypermutable region

p53 site-specific methylation status was measured in colonic DNA by quantitative PCR after digestion with *HpaII* (Fig. 3). Within the exons 5–8 region of *p53*, 2 *HpaII* sites were found in exon 6 and 7, respectively (Fig. 3a). The mean of ΔC_t is inversely related to the extent of methylation level at the *HpaII* cleavage site. The results demonstrate that the hypomethylation of exon 6 was induced to a marginally significant degree ($p = 0.05$) by the simultaneous inadequacies of vitamin B2, B6 and B12, whereas no effects even approaching significance were observed with the other depletion conditions. There were no significant differences of the *HpaII* site-specific methylation level on exon 7 (Fig. 3b).

p53 gene expression

As shown in Figure 4a, *p53* gene expression was numerically diminished in the groups with isolated folate depletion or folate combined with vitamin B2 or B6 depletion, but the decrease in expression was diminished to a significant degree ($p < 0.05$) only in 2 groups: the one with folate and B12 depletion and the one with multiple vitamin depletion. The electrophoresis results, which represent pooled RT-PCR products from 8 mice in each group, confirmed the real-time results (Fig. 4b).

Gene expression of *p53* downstream genes, *MDM2* and *p21*

The *p53* gene product is a transcription factor for the *MDM2* gene.⁴¹ Similarly, *p21* is a *p53*-inducible cyclin-dependent kinase inhibitor.⁴² Both *MDM2* and *p21* expression were measured to determine downstream effects of changes in *p53*. *MDM2* expression was modestly reduced to a nonsignificant degree ($p = 0.08$) by folate depletion alone when compared to the folate sufficient group, but was very substantially and significantly reduced, by a factor of 20-fold, by the combined vitamin depletion (Table IV). *MDM2* expression is significantly related to *p53* expression based on a Pearson correlation analysis ($p = 0.03$). No significant changes were observed for *p21* gene expression (data not shown).

Discussion

The current consensus of epidemiologic studies indicates that low folate status is associated with an increased risk for colorectal cancer development.¹³ Animal and cell culture studies indicate that probable mechanisms by which folate inadequacy enhances colorectal tumorigenesis include the induction of abnormal patterns of DNA methylation and the creation of obstacles in nucleotide synthesis,^{21,43–45} both of which are central functions of the biochemical network known as 1-carbon metabolism. However, besides folate, several other 1-carbon nutrients, including vitamin B2, B6 and B12, are integral cofactors in the 1-carbon nutrient metabolism network. Elucidation of the cellular mechanisms through which these 1-carbon nutrients cooperate to modulate tumorigenesis is important since marginal inadequacies of several B-vitamins are common in the industrialized countries.^{23–25} In our study, we demonstrate that simultaneous inadequacies of vitamins B2, B6 and B12 exaggerate the alterations in *p53* and one of its downstream effectors.

Prior rodent studies have shown that moderate or severe degrees of folate depletion can induce strand breaks in the hypermutable region of the *p53* gene.^{20,44} With the institution of folic acid fortification in North America and increasing recognition of significant shortfalls in nutritional adequacy in other B-vitamins, the importance of examining a very mild degree of folate depletion in conjunction with other inadequacies has therefore become more relevant. Our study demonstrates that several features of the *p53* gene are changed by mild depletion of folate combined with B2, B6 and B12. These findings indicate that molecular alterations that may not occur to a significant degree with mild folate depletion alone can be magnified by simultaneously imposing inadequacies of B2, B6 and B12. This emphasizes the synergy that may occur in the colon between mild insufficiency of folate and mild inadequacies of biochemically related nutrients.

With regard to gene integrity, the accumulation of DNA strand breaks within *p53* exons 6 and 8, but not exons 5 and 7, was significantly induced by mild folate depletion combined with inadequacies of vitamin B2, B6 and B12. Two previous studies^{20,21} in rats reported an induction of strand breaks due to folate deficiency but did not determine whether particular regions within this sequence of 4 exons are more susceptible than others. Our prior study²¹ indicates that the hypermutable region is more susceptible to these strand breaks than less

highly conserved regions, such as exons 2–4. Further studies will be needed to investigate whether similar site-specificity exists with multiple vitamin depletion.

The *p53* pathway is crucial for effective tumor suppression in humans and is composed of a network of genes that monitor and modulate DNA replication and repair, cell-cycle arrest and apoptosis.⁴⁶ Although our study shows that mildly diminished folate availability in conjunction with other 1-carbon nutrient inadequacies is capable of inducing anomalies in the *p53* gene, it is ultimately the demonstration of “downstream” effects that establishes the functional relevance of these phenomena on carcinogenesis. In our study, 2 genes whose expression is under control of *p53*, *MDM2* and *p21*, were interrogated. *MDM2* expression declined in association with diminished *p53* gene expression in the multiple vitamin depletion group, which is consistent with the stringency with which *p53* regulates *MDM2* expression.⁴⁷ *MDM2*, in turn is a negative feedback inhibitor of *p53* expression,⁴⁸ but how this negative feedback loop works in the context of multiple vitamin depletion was not clearly elucidated here. In contrast to *MDM2*, *p21* expression did not decrease in conjunction with diminished *p53* expression. *p21* expression is regulated on a transcriptional level by a variety of transcription factors, many of which operate *via p53*-independent pathways^{49,50} and therefore the absence of downregulation of *p21* in response to a change in *p53* is not entirely unexpected.

We recently reported that the same multiple vitamin depletion diet alters the expression of multiple components of the Wnt cascade in a manner that is consistent with increased Wnt-signaling.⁴⁰ It is important to recognize that the effects produced by aberrant *p53* expression that is reported here are not necessarily independent of those related to the Wnt cascade since it is thought that “cross-talk” exists between the 2 pathways^{51,52} For instance, although an increase in cellular β -catenin and increased translocation of the protein into the nucleus is commonly ascribed to impaired activity of upstream regulators in the Wnt-signaling pathway, it is also been shown to occur as a result of *p53* inactivation.⁵¹ Thus, the effects that the multiple vitamin depletion diet have on the cell cycle of the colonic epithelium that we have previously reported,⁴⁰ such as impaired apoptosis, may be a consequence of dysregulated Wnt- or *p53*-signaling, or perhaps an integrated outcome of both.

As reported in an earlier publication, no macroscopic or microscopic neoplasms were found in our study despite a systematic search for these lesions.⁴⁰ This was entirely consistent with our expectations: epidemiological as well as animal studies indicate that folate depletion merely enhances an underlying predisposition towards tumorigenesis and is an insufficient factor by itself to initiate the development of neoplasms.^{53,54} However, our study indicates mild folate depletion combined with insufficiencies of B2, B6 and B12 alters the methylation, integrity and expression of the *p53* gene as well as the expression of a gene under stringent control of *p53*. The creation of such a molecular environment could, in combination with other genetic and environmental factors, impart a potent propensity toward carcinogenesis.

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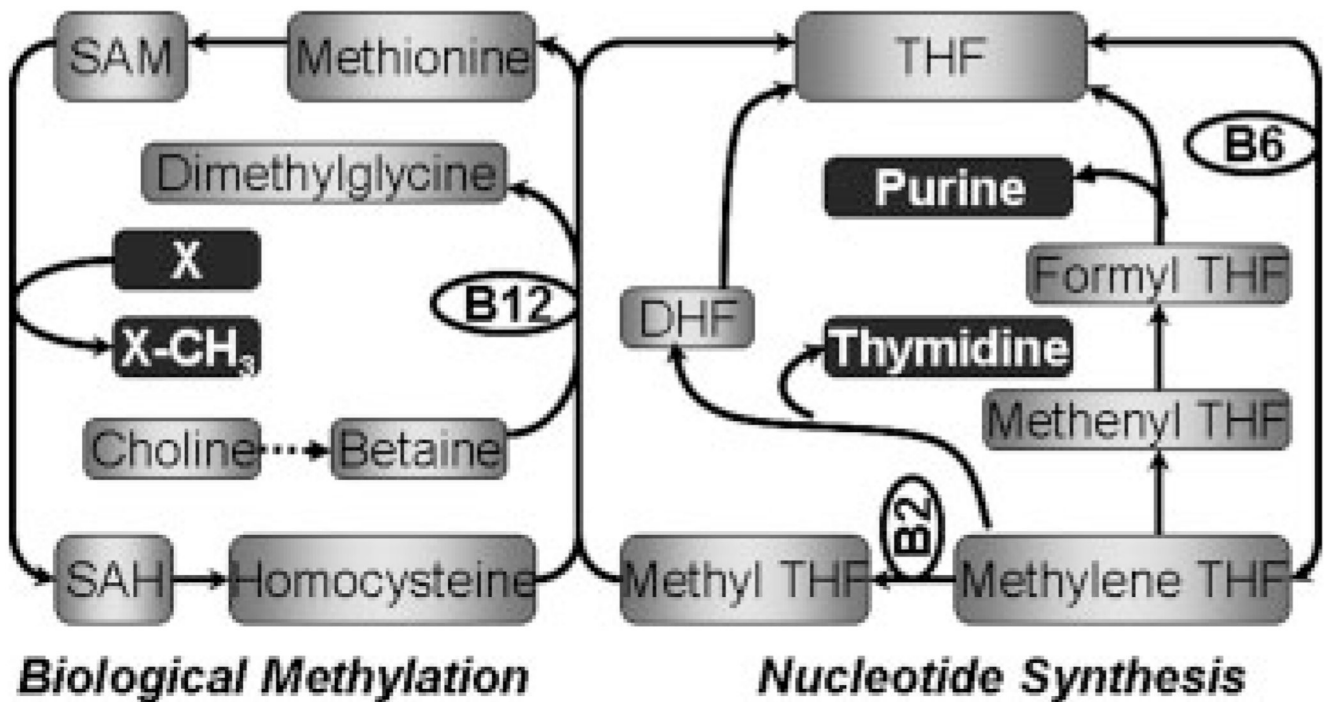
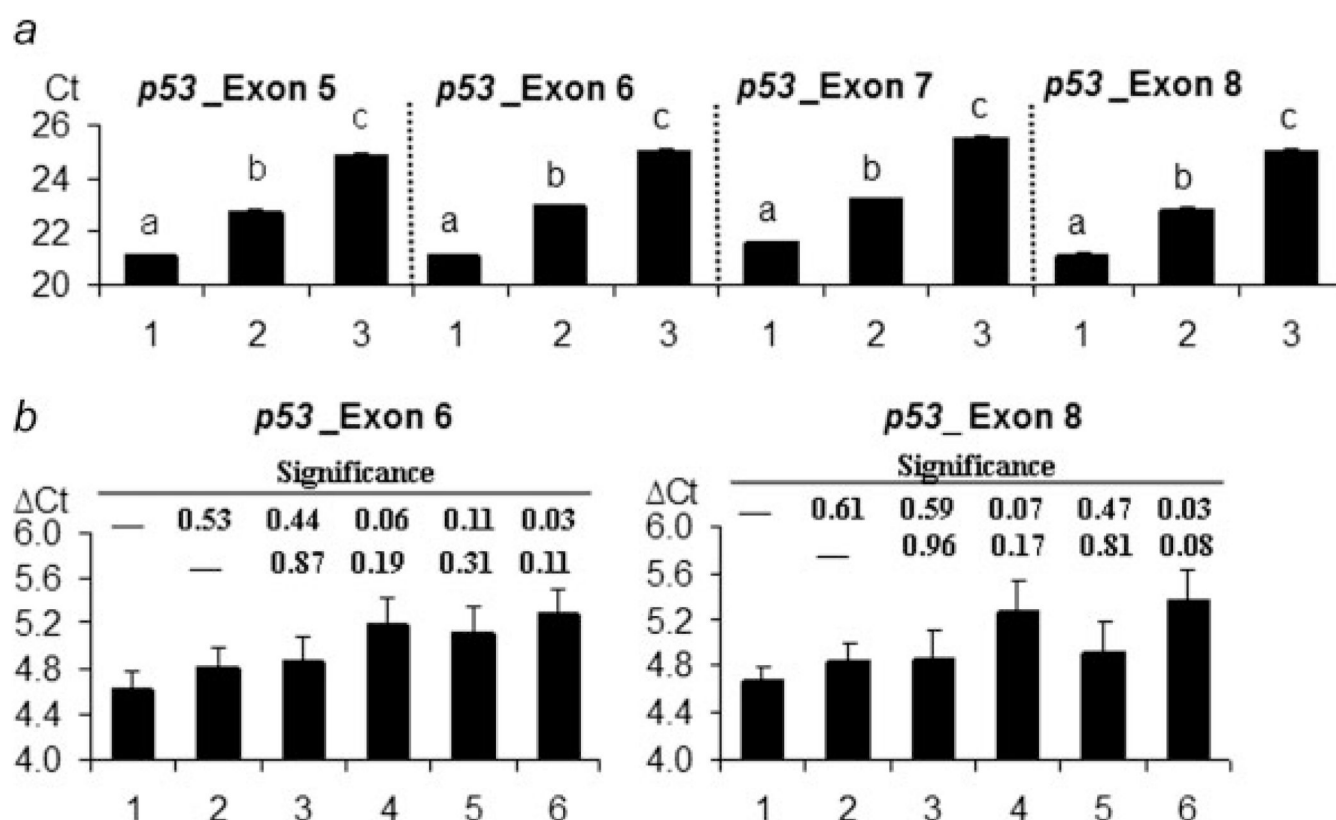


FIGURE 1.

The roles of folate and other B-vitamins (B2, B6 and B12) in biological methylation and nucleotide synthesis.^{5,6} SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; THF, tetrahydrofolate; DHF, dihydrofolate; X, molecules which may be methylated.

**FIGURE 2.**

The effect of B-vitamin depletion on DNA strand breaks within the *p53* gene, exons 5–8. (a) Primer design for *p53* exons 5–8. Primer sets were designed for each of the 4 exons from 5 to 8. The reaction cycle thresholds (Ct) were incrementally increased ($p < 0.01$) with the percentage of DNA templates for all 4 primer sets. Template DNA concentration in the reaction mixtures: 1, 10 ng/μl; 2, 2.5 ng/μl; 3, 0.625 ng/μl. (b) Multiple B-vitamin depletion significantly magnified strand breaks in exons 6 and 8 compared to the folate sufficient group whereas there were no significant differences in strand breaks in exons 5 and 7 among the different dietary groups (data not shown). The top row of significance values represents p -values compared to the folate sufficient group and the second row are the p -values compared to the folate deficient group. The data in the figure are expressed as mean $\pm$ SEM and the sample size is $n = 8$ /dietary group. 1, folate sufficiency; 2, folate depletion; 3, folate and B2 depletion; 4, folate and B6 depletion; 5, folate and B12 depletion; 6, multiple vitamin depletion.

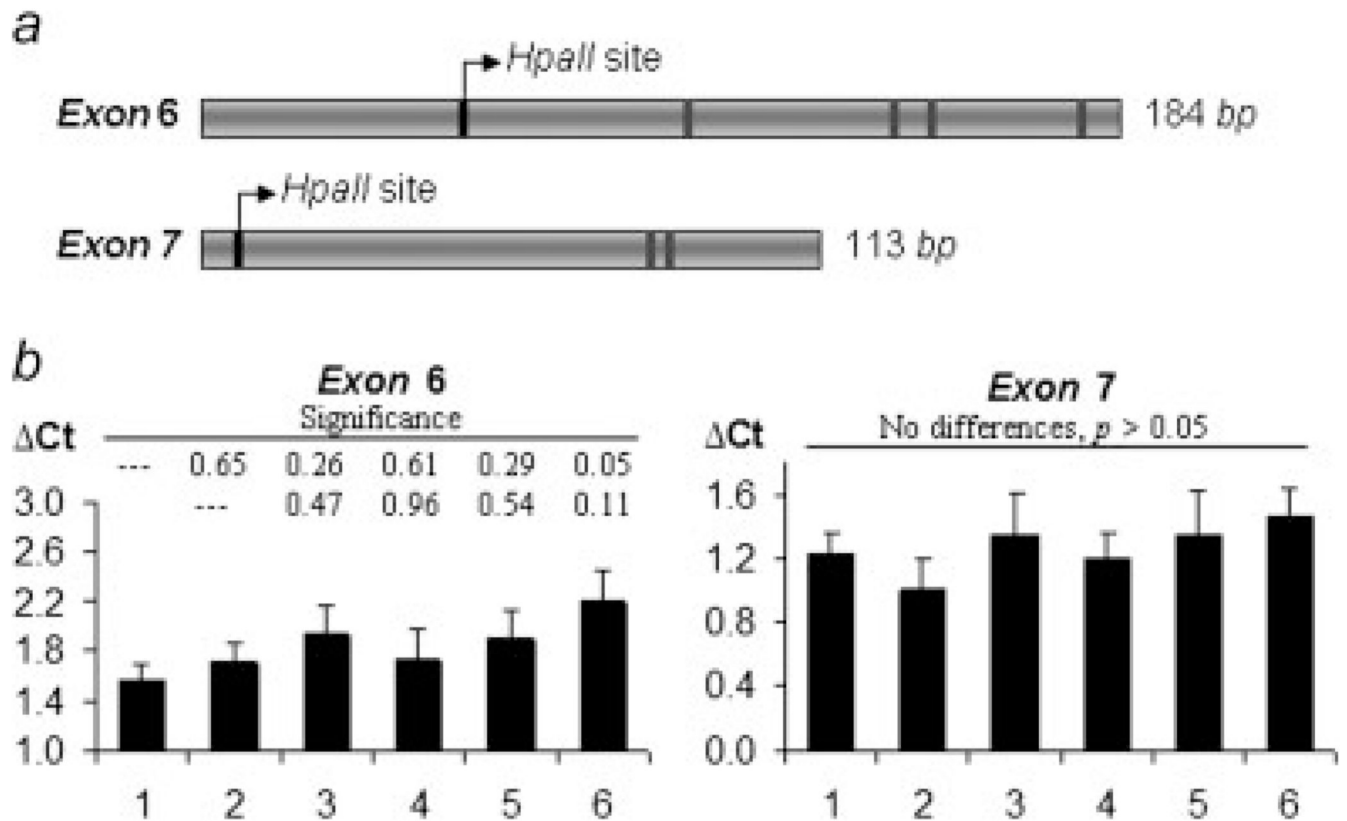


FIGURE 3.

The effect of B-vitamin depletion on DNA methylation in *p53* gene, exons 6 and 7. (a) Exon 6 has a length of 184 bp with 5 CpG sites and 1 *HpaII* site and exon 7 has a length of 113 bp with 3 CpG sites and 1 *HpaII* site. The cleavage sites by the methyl-sensitive *HpaII* restriction enzyme are shown. (b) A significant degree of DNA hypomethylation developed in exon 6 in the multiple B-vitamin depletion group compared to the folate sufficient group. The top row of significance values represents the p -values compared to the folate sufficient group and the second row are the p -values compared to the folate deficient group. The data in the figure are expressed as mean $\pm$ SEM and the sample size is $n = 8$ /dietary group. 1, folate sufficiency; 2, folate depletion; 3, folate and B2 depletion; 4, folate and B6 depletion; 5, folate and B12 depletion; 6, multiple vitamin depletion.

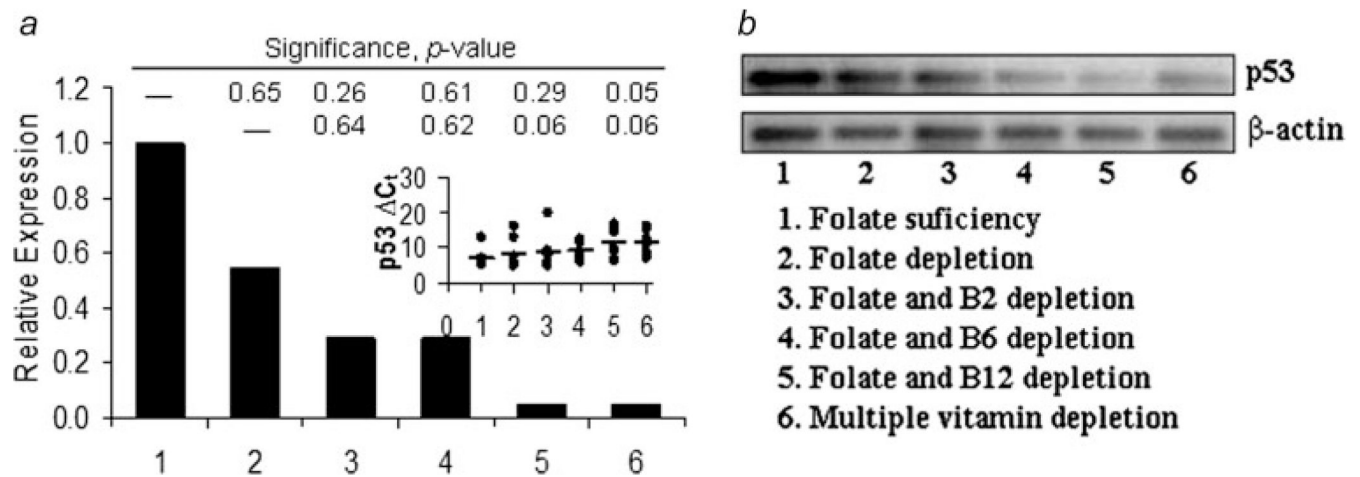


FIGURE 4.

Effect of vitamin depletion on *p53* gene expression. (a) The data analysis is based on *p53* ΔC_t value normalized to β -actin, as measured by real-time PCR. The top line of p -values indicates the significance compared to the folate sufficient group and second line indicates the significance compared to folate depletion group. (b) Mean of *p53* expression, as determined by reverse-transcriptase (RT)-PCR. The density of the RT-PCR bands on the gel represents the average *p53* gene expression with cDNA pooled from 8 mice in each group. The data in the figure are expressed as mean $\pm$ SEM and the sample size is $n = 8$ /dietary group.

TABLE I
Composition of vitamin mix¹

Ingredient (g/kg)	Folate sufficiency	Folate depletion	Folate and B2 depletion	Folate and B6 depletion	Folate and B12 depletion	Multiple vitamin depletion
Thiamin HCl	0.6	0.6	0.6	0.6	0.6	0.6
Riboflavin	0.6	0.6	0.05	0.6	0.6	0.05
Pyridoxine HCl	0.7	0.7	0.7	0.05	0.7	0.05
Nicotinic acid	3.0	3.0	3.0	3.0	3.0	3.0
Calcium pantothenate	1.6	1.6	1.6	1.6	1.6	1.6
Vitamin B12 (0.1%)	5.0	5.0	5.0	5.0	0.0	0.0
Vitamin A palmitate (500,000 U/g)	0.8	0.8	0.8	0.8	0.8	0.8
Vitamin D3 (400,000 U/g)	0.25	0.25	0.25	0.25	0.25	0.25
Vitamin E acetate (500 U/g)	10.0	10.0	10.0	10.0	10.0	10.0
Menadione sodium bisulfite	0.08	0.08	0.08	0.08	0.08	0.08
Biotin	0.02	0.02	0.02	0.02	0.02	0.02
Folic acid	0.20	0.00	0.00	0.00	0.00	0.00
Sucrose, finely powdered	977.15	977.35	977.90	978.00	982.35	983.55
Total	1,000	1,000	1,000	1,000	1,000	1,000

¹The basal diet is an amino-acid-defined diet based on Clifford folate deficient diet. This diet is modest in fat (22.5% of total calories), adequate in fiber (50 g/kg of diet) and contains adequate amounts of vitamin D and calcium (1,000 IU of vitamin D and 5.1 g of Ca/kg of diet, respectively). This basal diet contains 8.03 g of methionine and 2.02 g of choline chloride/kg of diet. The vitamin mix was added as 1% for each group.

TABLE IIPrimer sequences and pcr conditions for *p53* exons 5–8 and β -actin

Primer set	Sense primers	Antisense primers	Amp. size (bp)	T_m^1 (°C)
Exon 5	5'-CCCACCTTGACACCTGATCG-3'	50'-ACCATCACCATCGGAGCAG-3'	251	60/59
Exon 6	5'-TTCTGACTTATTCCTTGCTCTTAGGCC-3'	5'-AACAAAATTACAGACCTCGGGTG-3'	151	59/58
Exon 7	5'-CTGCTGCAGGTCACCTGTAGTG-3'	5'-GGTAAGGATAGGTCGGCGGT-3'	151	59/60
Exon 8	5'-AGTGGGAACCTTCTGGGACG-3'	5'-GGTGAAGCTCAACAGGCTCCT-3'	286	60/59
β -actin	5'-TGCTGTCCCTGTATGCCTCTG-3'	5'-TCCATCACAATGCCTGTGGTAC-3'	46	60/59

¹ Melting temperature for sense and antisense primers.

TABLE III
Vitamin status in blood and tissue and genomic dna methylation¹

Treatment	Plasma folate (µg/l)	RBC riboflavin (act. coeff.) ²	Plasma vitamin B-6 (nmol/l)	Plasma vitamin B-12 (µg/l)	Plasma homocysteine (µmol/l)	Colonic folate (nmol/g)
Folate sufficiency	68.08 ± 3.72 ^a	1.21 ± 0.02 ^c	184.39 ± 9.78 ^a	17.56 ± 1.25 ^a	11.39 ± 0.76 ^b	1.23 ± 0.10 ^a
Folate depletion	31.90 ± 4.05 ^c	1.24 ± 0.03 ^b	198.26 ± 12.22 ^a	13.62 ± 1.27 ^b	16.20 ± 1.57 ^a	0.83 ± 0.10 ^b
Folate and B-2 depletion	32.85 ± 3.06 ^c	1.33 ± 0.04 ^a	176.93 ± 11.54 ^a	17.41 ± 1.12 ^a	13.28 ± 1.45 ^{a,b}	0.66 ± 0.09 ^b
Folate and B-6 depletion	43.75 ± 3.06 ^b	1.21 ± 0.02 ^c	70.58 ± 6.91 ^b	19.45 ± 1.80 ^a	15.13 ± 1.59 ^a	0.76 ± 0.10 ^b
Folate and B-12 depletion	31.83 ± 3.11 ^c	1.23 ± 0.02 ^b	180.26 ± 9.66 ^a	6.08 ± 0.31 ^c	13.75 ± 0.77 ^{a,b}	0.71 ± 0.08 ^b
Multiple vitamin depletion	43.37 ± 3.39 ^b	1.29 ± 0.02 ^{a,b}	81.64 ± 9.92 ^b	5.92 ± 0.50 ^c	16.03 ± 1.51 ^a	0.73 ± 0.10 ^b

^{a-c}Values with the same superscript letter indicate no significant differences among groups in the same column at $\alpha = 0.05$ level.

¹Data are mean ± SEM; $n = 8$ /dietary group.

²High activation coefficients indicate low systemic B2 status.

TABLE IV

Expression of the *MDM2* gene¹

Treatment	ΔCt^2	Relative expression
Folate sufficiency	9.84 ± 0.99 ^a	1.00
Folate depletion	12.48 ± 0.99 ^{a,b}	0.16
Folate and B2 depletion	11.01 ± 0.83 ^{a,b}	0.44
Folate and B6 depletion	12.80 ± 1.20 ^{a,b}	0.13
Folate and B12 depletion	12.13 ± 0.70 ^{a,b}	0.20
Multiple vitamin depletion	14.16 ± 1.21 ^b	0.05

^{a,b} Values with the same superscript letter indicate no significant differences among groups at $\alpha = 0.05$ level.

¹ Data are mean ± SEM; $n = 8$ /dietary group.

² The expression of *MDM2* was normalized to β -actin ($\Delta Ct = Ct_{MDM2} - Ct_{\beta\text{-actin}}$).