Older Age and Dietary Folate Are Determinants of Genomic and p16-Specific DNA Methylation in Mouse Colon

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Abstract
Older age and inadequate folate intake are strongly implicated as important risk factors for colon cancer and each is associated with altered DNA methylation. This study was designed to determine the effects of aging and dietary folate on select features of DNA methylation in the colon that are relevant to carcinogenesis. Old (18 mo; n = 34) and young (4 mo; n = 32) male C57BL/6 mice were randomly divided into 3 groups and fed diets containing 0, 4.5, or 18 μmol folate/kg (deplete, replete, and supplemented groups, respectively) for 20 wk. Genomic DNA methylation and p16 promoter methylation in the colonic mucosa were analyzed by liquid chromatography/electrospray ionization/MS and methylation-specific PCR, respectively. p16 gene expression was determined by real-time RT-PCR. Old mice had significantly lower genomic DNA methylation compared with young mice at each level of dietary folate (4.5 ± 0.2, 4.8 ± 0.1, and 4.9 ± 0.1 vs. 6.0 ± 0.1, 5.3 ± 0.2, and 5.9 ± 0.2%, in deplete-replete, -replete, and -supplemented groups, respectively, P < 0.05) and markedly higher p16 promoter methylation (61.0 ± 2.7, 69.7 ± 6.9, and 87.1 ± 13.4 vs. 10.8 ± 3.6, 8.4 ± 1.8, and 4.9 ± 1.7%, respectively, P < 0.05). In old mice, genomic and p16 promoter DNA methylation each increased in a manner that was directly related to dietary folate (R2 = 0.009). Age-related enhancement of p16 expression occurred in folate-replete (P = 0.001) and folate-supplemented groups (P = 0.041), but not in the folate-deplete group. In conclusion, aging decreases genomic DNA methylation and increases promoter methylation and expression of p16 in mouse colons. This effect is dependent on the level of dietary folate. J. Nutr. 137: 1713–1717, 2007.

Introduction
DNA methylation, an epigenetic phenomenon, is a major determinant of gene expression and integrity. DNA methylation occurs via specific methyltransferases, which catalyze the transfer of a methyl group from S-adenosylmethionine to the carbon-5 position of cytosine in CpG dinucleotides (1). Methylation within genes or gene regulatory elements such as exons and promoters generally represses transcription of the gene, whereas methylation within gene-deficient regions is involved in the conformation and integrity of the chromosome (2,3).

As differentiation approaches completion during early development, tissue-specific patterns of methylation are acquired that are stably maintained during the lifetime of the organism (4). However, these patterns are not immutable; aging and nutritional factors have been shown to modify patterns of DNA methylation in mammalian tissues (5,6). Recent observations suggest that some of these altered patterns of methylation may have pathologic consequences, contributing to cellular hyperproliferation associated with aging and the development of malignancies (7,8). Interestingly, malignancies usually express diminished genomic DNA methylation and concurrent hypermethylation of several critical genes. The latter is thought to be an important avenue by which the expression of tumor suppressor genes is attenuated in neoplasia (9,10). Aging can alter DNA methylation, which has the potential to promote age-related conditions such as cancer (11).

Aging changes DNA methylation in a complex fashion (12). In general, genomic DNA methylation tends to decrease with aging (11). In a previous rodent study, brain, liver, small intestinal mucosa, heart, and spleen all exhibited genomic DNA hypomethylation with aging. Conversely, the kidney demonstrated hypermethylation, whereas the lung showed no changes (13,14). Aging also affects gene-specific promoter DNA methylation (15,16). Age-dependent methylation changes in CpG islands, where methylation correlates strongly with the suppression of...
gene expression, have been the subject of multiple recent reports (17). For example, Issa et al. (16) reported that in young individuals, CpG islands in the promoter region of the colonic estrogen receptor gene are not methylated. Meanwhile, partial methylation appears in older individuals and the region is invariably hypermethylated in colonic adenomas and cancers.

The p16 gene product is a tumor suppressor that participates in the regulation of the cell cycle, retarding proliferation. Loss of p16 function by promoter methylation is a common epigenetic phenomenon in human colorectal carcinogenesis (18,19). Also, expression of the gene, which generally increases with aging (20), is thought to play a mechanistic role in the aging process, because it promotes cell senescence and age-related decline in the functions of some organs (21,22). Therefore, modifying p16 gene expression by altering promoter methylation through dietary means may feasibly affect the aging process as well as carcinogenesis (23,24).

The 1-carbon group that is transferred to DNA for methylation is largely derived from methylated folate. Therefore, it is not surprising that folic acid inadequacy has a large impact on DNA methylation in both humans and animals, an affect that is hypothesized to explain the relationship between folate status and the risk of colon cancer (10). Recently we found that older age produces both quantitative and qualitative changes in folate metabolism in the rodent colon, resulting in limited availability of the vitamin in this tissue. Modest levels of dietary folate supplementation were sufficient to reverse these age-related derangements (25). This suggests that aging might enhance colonic carcinogenesis, at least in part, through alterations in folate metabolism, but this also indicates that this process can be ameliorated by a nutritional intervention.

In this study, we examined the epigenetic effect of aging in the mouse colon by measuring genomic and p16 promoter DNA methylation and altered expression of this gene. We further examined how folate adequacy modulates this relationship between age and DNA methylation in the colon.

### Materials and Methods

#### Animals and diets

This study was approved by the Institutional Animal Care and Use Committee of the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University. Old (18 mo) male C57BL/6 mice (n = 34) and young (4 mo) mice (n = 32) were randomly divided into 3 groups and fed Walzem and Clifford amino acid-defined diets (26) containing 0, 4.5, or 18 μmol folic acid/kg (deplete, replete, and supplemented groups, respectively) for 20 wk. All diets were obtained from Dyets. No sulfonamide antibiotics were used. The mice were housed individually, body weights were recorded every other week, and they consumed water ad libitum. The mice were group pair-fed to decrease variability in food, energy, and nutrient intakes within and between dietary groups; all mice in each age group were given a measured portion of food daily. When any one mouse did not eat its entire portion of food, the next day’s portion was decreased to the amount that was eaten by that mouse the previous day. When all the mice consumed all of their allocation, the quantity of diet given daily was increased until the mice stopped consuming the given amount.

At 20 wk after initiation of the diets, all mice from each group were killed. Mice were anesthetized with isoflurane and blood was drawn via cardiac puncture. The abdomen was then opened and the colorectum was excised and opened longitudinally on an ice-cold glass plate. After being rinsed with ice-cold saline, the mucosa was gently scraped off with glass microscope slides, as previously described (25). Aliquots of mucosal scraping were flash-frozen in liquid nitrogen and subsequently stored at −70°C. Plasma folate concentration was determined by a competitive immunoassay method using a commercially available kit (Immulse, DPC).

#### Genomic DNA methylation

DNA methylation was determined using liquid chromatography/electrospray ionization MS as described (27). Briefly, 1 μg of DNA was hydrolyzed by sequential digestion with 3 enzymes: nucleases P1 (Roche Molecular Biochemicals), venom phosphodiesterase I (Sigma), and alkaline phosphatase (Sigma). The hydrolyzed DNA solution was directly delivered onto the analytical column (Supelco) in isocratic mode (Hewlett Packard series 1100). This allowed the separation of the 4 DNA bases and the identification of 5-methylcytosine. Electrospray ionization MS (Hewlett Packard) was performed in positive ion mode. Identification of cytosine and 5-methylcytosine was obtained by MS analysis of chromatographic peaks. The isopentomers 15N2, 2-deoxycytidine and methyl-D3, ring-6-D1 5-methyl-2'-deoxycytidine (Cambridge Isotope Laboratories) were used as internal standards. DNA methylation status was defined as the percentage of cytosine and 5-methylcytosine that was in the latter form.

#### Promoter methylation

DNA methylation of the CpG islands of the p16 promoter region was determined by bisulfite modification of genomic DNA and subsequent methylation-specific PCR, as described in detail elsewhere (28,29).

Briefly, 1 μg of genomic DNA was denatured in 0.3 mol/L NaOH at 37°C for 15 min and the bisulfite reaction was carried out in 2 mol/L sodium metabisulfite at 55°C for 16 h. DNA was recovered by a desalting column (Promega) and desulfonated in 0.2 mol/L NaOH at 37°C for 15 min, neutralized by ammonium acetate, alcohol precipitated, dried, and then resolved in tris-EDTA buffer. DNA was amplified with a methyl-specific primer set (M forward, CGATGGGCGGTATTGTG-AATTTCGCC; M reverse, CAGCTCATACAGCACCTTAAACCG) and an unmethyl-specific primer set (U forward, GTGATTGGGTGG-GATTGAATTTTGTG; U reverse, CCACATCATACACACACC-TAAAACA) for the mouse p16 promoter site (29). Methyl amplification conditions were as follows: initial denaturation at 95°C for 10 min, denaturation at 94°C for 30 s, annealing at 66°C for 1 min, and extension at 72°C for 1 min for 40 cycles, followed by a stabilization for 8 s at 72°C. Unmethyl amplification followed the same procedure with the exception of the annealing temperature, which was 64°C. Amplification products were separated by gel electrophoresis and stained with ethidium bromide. The density (intensity × square millimeters) of each band specific for methyl and unmethyl primers was measured under UV light and promoter methylation status was expressed by percent density of methyl band/[methyl band + unmethyl band] (30).

#### Real-time RT-PCR assay for p16 gene expression

Total cellular RNA was isolated from colonic mucosa with Trizol reagent (Invitrogen) and the first strand of cDNA was synthesized using oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen). The expression of the p16 gene was quantified using a Taqman Gene Expression Assay and an ABI Prism 7300 Sequence Detection system (Applied Biosystems). Gene expression was normalized to GAPDH (ΔCt = CpC 16−CpC GAPDH) and statistical analyses were performed using ΔCt. The lower ΔCt represents the higher p16 gene expression.

#### Statistics

The primary analytic method in this study was 2-way ANOVA to examine the effect of aging as well as diets. Post hoc analysis with Bonferroni’s method was used to determine the locations of differences after ANOVA. Regression analysis was used for determining the folate dose effect (P trend). The level of significance was set at P < 0.05 for all analyses.

### Results

The body weights of old mice were stable throughout the experiment with the exception of the folate-supplemented mice, who sustained a modest weight loss during the last month of the experiment. At 16 and 18 wk of the study, the body weight of the folate-supplemented old group was less than that of the other 2 aged groups (Fig. 1). In contrast, the body weights of young mice increased progressively during the 20 wk and the 3 dier groups...
did not differ from one another. During the 20 wk, 2 young and 3 old mice died. No specific abnormalities or disease processes were identified at necropsy in these 5 mice. Plasma folate concentrations increased in a dose-dependent fashion from the deplete group to the supplemented group in both old (P < 0.001) and young mice (P = 0.002). Plasma folate concentrations did not differ between old and young mice in each diet group (Table 1).

Old mice had lower genomic DNA methylation in the colon compared with corresponding young mice in each diet group (folate deplete, P < 0.001; folate replete, P = 0.078; folate supplemented, P < 0.001). In the old mice, genomic DNA methylation increased in a manner that was directly related to dietary folate (P trend < 0.023), whereas this association was not at all evident in the young mice (Table 2). At each level of dietary folate, old mice demonstrated a markedly greater degree of p16 promoter methylation compared with the corresponding young groups (P < 0.001, Fig. 2). As was the case with genomic methylation, p16 methylation in the young mice was not altered by folate availability. In contrast, old mice showed a stepwise increase in p16 promoter methylation across the spectrum of dietary folate (P trend = 0.009) (Fig. 2).

Old mice fed the folate-replete (P = 0.001) and folate-supplemented (P = 0.041) diets had greater p16 expression (lower ΔCt) compared with young mice fed the same diets (Table 3).

### Discussion

This study demonstrates diminished genomic and increased p16 promoter DNA methylation in the colon of aged mice compared with that of young mice and a dependency of both phenomena on the level of dietary folate. On the contrary, in the young mice, neither genomic nor p16 promoter methylation was susceptible to modulation by dietary folate. This recapitulates an earlier study in which we showed that the older rodent colon is particularly vulnerable to the induction of a molecular aberration induced by limited folate availability, which in the case of the earlier study was uracil misincorporation in DNA (23). This emphasizes the idea that alterations in folate availability in humans may have a greater impact on colonic carcinogenesis in elder adults compared with younger people. To the best of our knowledge, this is the first study to examine how aging and folate interact to determine genomic and promoter DNA methylation in rodent colon.

Our observations of increased promoter methylation and expression of the p16 gene in the colon of aged mice contrasts somewhat with other studies (31–33) whose results have indicated that p16 promoter methylation represses this gene. However, several studies have shown that promoter methylation is not the sole determinant of p16 expression (34–36) and we

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### Table 1

<table>
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<tr>
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*P trend = 0.002 <0.001

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### Table 2

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<td>n</td>
<td>%</td>
</tr>
<tr>
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<td>4.5 ± 0.2</td>
</tr>
<tr>
<td>FR</td>
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<td>4.8 ± 0.1</td>
</tr>
<tr>
<td>FS</td>
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<td>4.9 ± 0.1</td>
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*P trend = 0.023 NS

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### Table 3

<table>
<thead>
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<th>%</th>
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<th>%</th>
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<tr>
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<td>9</td>
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*P trend = 0.009 NS, non-significant.
TABLE 3  p16 Gene expression in old (18 mo) and young (4 mo) male C57BL/6 mice fed diets containing 0 (FD), 4.5 (FR), or 18 (FS) μmol folic acid/kg diet for 20 wk

<table>
<thead>
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<th>Diet</th>
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<th>Young mice</th>
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<tr>
<td>n</td>
<td>ΔΔCt</td>
<td>n</td>
</tr>
<tr>
<td>FD</td>
<td>12</td>
<td>16.2 ± 0.6</td>
</tr>
<tr>
<td>FR</td>
<td>10</td>
<td>16.2 ± 0.4</td>
</tr>
<tr>
<td>FS</td>
<td>12</td>
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Values are means ± SEM. *Different from old mice, P < 0.05. ΔΔCt = Ct<sub>p16</sub> - Ct<sub>Gapdh</sub>.

speculate that other factors associated with aging must predominate over any effect that promoter methylation may have on p16 expression in the colon of aged mice. Although our observation of high p16 expression in the setting of high levels of promoter methylation is consistent with observations made in a few other genes, such as the human telomerase catalytic subunit gene (37), it is counter to the current theory that hypermethylation of CpG islands in select promoter regions is associated with transcriptional silencing of gene expression in carcinogenesis (31–33). This discrepancy may be due to different characteristics of promoter methylation induced by aging and that which is induced by carcinogenesis. In carcinogenesis, hypermethylation is associated with aberrant DNA methyltransferases expression (38) and concurrent abnormal changes in histone acetylation and methylation (39), which are known to repress critical genes along with promoter DNA methylation (40). The effect of partial methylation on gene expression in the physiologic condition of advancing age may be different from that of the fully methylated promoter methylation of cancer (34–36). It is also possible that the methylation status of the specific CpG residues targeted by our assay is not indicative of the overall methylation status of the p16 promoter. This issue requires further investigation.

We previously showed that aging is associated with a particular vulnerability to folate depletion, especially in organs such as the colon. Perhaps because of this phenomenon, certain biochemical processes dependent on folate (such as DNA methylation and uracil incorporation) display a marked sensitivity to dietary folate availability in older animals (25,41). This study again demonstrates this effect in regard to both genomic and p16 methylation in the colon. The exact mechanism by which aging reduces genomic DNA methylation and the physiological importance of reduced genomic DNA methylation have not yet been elucidated. However, it has been hypothesized that aging, a strong risk factor for cancer, establishes an epigenetic environment conducive to cancer development by reducing genomic DNA methylation or increasing promoter methylation of tumor suppressor genes (42), because these alterations are frequently found in cancer and are regarded by many as important causal mechanisms (10). Thus, investigation into the effect of aging as well as the synergistic effect of folate status may be important for our understanding of colon carcinogenesis and chemoprevention of colon cancer (43).

In contrast to genomic methylation, p16 promoter methylation was significantly increased in old mice. This observation is consistent with a previous report that investigated global patterns of CpG island methylation in normal human colon and found progressive methylation of promoters in an age-dependent manner (44). However, the results have not been entirely consistent in the various studies; in other human studies, p16 promoter methylation was positively associated with aging in the stomach (45), but another study did not observe such an effect in the colon (46). Interestingly, in our study, increasing levels of dietary folate increased promoter methylation, mimicking what occurred with genomic DNA methylation. Thus, increasing levels of dietary folate over the range that we studied can increase both genomic and promoter methylation in old, but not young, mice. To the extent that these methylation phenomena affect the aging process or carcinogenesis, our observations suggest that dietary folate might affect these events more dramatically in older than in younger adults.

The observation that p16 gene expression increased in old mice is consistent with previous reports. The expression of p16 increases markedly in almost all rodent tissues with advancing age (21). p16 expression in the colon, which is very low in healthy mucosa, increases in the earliest stages of carcinogenesis and correlates inversely with cell proliferation (47). Thus, the tumor suppressor function of p16 may be particularly important in determining where there is a strong underlying predisposition to cancer (i.e. aging), or where an incipient neoplasm is already present. In this study, colonic p16 expression was greater in old than in young folate-replete and folate-supplemented mice but not in those fed the folate-deplete diet. This may indicate that adequacy of dietary folate is important for maintaining sufficient expression of p16 in the aged colon Table 3.

During the 20 wk of this study, the young mice showed a progressive weight gain regardless of dietary folate level. The body weights of old mice were unchanged except for the folate-supplemented group, which lost a small but significant portion of their weight at the end of study. This was an unexpected observation, especially under the conditions of group pair-feeding, and is not a phenomenon we have observed in our prior studies. A recent rodent study demonstrated that long-term high-folate supplementation reduces nitrogen digestive function in aged rats, indicating that folate supplementation at high doses could have a negative effect on protein utilization (48). Other rodent studies have reported that fetuses from folate-supplemented dams had significantly reduced body weight compared with fetuses from control dams (49,50). These observations offer feasible explanations for what we observed, but no other evidence suggests that folate supplementation reduces body weight in old mice.

In brief, our observations indicate that aging reduces genomic DNA methylation and increases p16 promoter methylation in mouse colons. Folate supplementation, which can enhance methyl availability, increases both genomic DNA methylation and p16 promoter methylation in old mice. This epigenetic change by aging and dietary folate affects the expression of p16, a critical gene for both aging and carcinogenesis.

**Literature Cited**


Myohanen SK, Baylin SB, Herman JG. Hypermethylation can selectively silence individual p16ink4a alleles in neoplasia. Cancer Res. 1998;58:30–8.


