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Mechanisms of DNA Damage, DNA Hypomethylation, and Tumor Progression in the Folate/Methyl-Deficient Rat Model of Hepatocarcinogenesis[1]

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ABSTRACT Using the folate/methyl-deficient rat model of hepatocarcinogenesis, we obtained evidence that may provide new insights into a major unresolved paradox in DNA methylation and cancer research: the mechanistic basis for genome-wide hypomethylation despite an increase in DNA methyltransferase activity and gene-specific regional hypermethylation. Previous studies revealed that the methyltransferase binds with higher affinity to DNA strand breaks, gaps, abasic sites, and uracil than it does to its cognate hemimethylated CpG sites, consistent with its ancestral function as a DNA repair enzyme. These same DNA lesions are an early occurrence in models of folate and methyl deficiency and are often present in human preneoplastic cells. We hypothesized that the high-affinity binding of the maintenance DNA methyltransferase to unrepaired lesions in DNA could sequester available enzyme away from the replication fork and promote passive replication-dependent demethylation. In support of this possibility, we found that lesion-containing DNA is less efficiently methylated than lesion-free DNA from folate/methyl-deficient rats and that an increase in DNA strand breaks precedes DNA hypomethylation. Despite an adaptive increase in DNA methyltransferase activity, hemimethylated DNA from folate/methyl-deficient rats is progressively replaced by double-stranded unmethylated DNA that is resistant to remethylation with dietary methyl repletion. In promoter regions, the inappropriate binding of the DNA methyltransferase to unrepaired lesions or mispairs may promote local histone deacetylation, methylation, and regional hypermethylation associated with tumor suppressor gene silencing. These insights in an experimental model are consistent with the possibility that DNA lesions may be a necessary prerequisite for the disruption of normal DNA methylation patterns in preneoplastic and neoplastic cells. J. Nutr. 133: 3740S–3747S, 2003.

KEY WORDS: • *folate deficiency* • *methyl deficiency* • *DNA methylation* • *hepatocarcinogenesis* • *DNA strand breaks* • *uracil* • *deoxynucleotides*

Tumor induction with dietary methyl-donor deficiency was first observed in 1946 by Copeland and Salmon (1) using a semisynthetic choline-devoid diet that was low in methionine and folic acid. Subsequently, the strong promoting activity of the diet after initiation by a variety of chemical carcinogens was demonstrated by Newberne and others (2,3). In 1983 it was definitively demonstrated that diets lacking in methionine and choline and without detectable levels of carcinogens could act independently as a complete carcinogen (4,5). The present consensus, therefore, is that diets low in methionine and devoid of choline and folate are sufficient to independently induce tumor formation in rats, primarily in the liver (6). The methyl-deficient model of endogenous carcinogenesis is unique in that dietary omission rather than xenobiotic addition leads to tumor formation. Thus, the biochemical and molecular events predisposing to cancer in this model are a result of chronic metabolic stress and provide an ideal model system in which to study progressive molecular and metabolic alterations that occur during tumor progression.

Because of the metabolic interdependency among methionine, choline, and folate, a deficiency in any of the three nutrients will perturb the metabolic priorities of the other two (7). Briefly, when exogenous methionine and choline are limited in the diet, the folate-dependent pathway for the endogenous resynthesis of methionine from homocysteine is upregulated as a mechanism to maintain intracellular levels of

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S-adenosylmethionine (SAM)[3], the major intracellular methyl donor. The diet-induced decrease in SAM levels has an indirect effect on folate (tetrahydrofolate, THF) metabolism by inducing feedback stimulation (derepression) of 5,10-methylene-THF reductase and the consequent diversion of 5,10-methylene-THF to 5-methyl-THF (8). As a result of this diversion, the methyl group from 5-methyl-THF is transferred to homocysteine (via methyl-B-12), thereby promoting the endogenous resynthesis of methionine and, subsequently, SAM. It is important to note that the increased turnover of this pathway, stimulated by decreased availability of SAM, effectively increases the intracellular requirement for folate (9) and can lead to a 30–50% decrease in liver folate content (10). The indirect consequence of the irreversible diversion of folate methyl groups toward the regeneration of methionine (the well-established methyl trap) is a functional depletion of folate 1-carbon groups for the de novo synthesis of purines and thymidylate (7). As a result, inadequate folate availability will negatively affect DNA metabolism and integrity with genetically significant consequences. The central importance of folate-mediated 1-carbon transfer for the maintenance of both de novo synthesis of deoxynucleotides and SAM-dependent DNA methylation is presented in **Figure 1**.

Because the deoxynucleotide triphosphates (dNTPs) are the substrate for the DNA polymerases, the fidelity of DNA replication and repair synthesis is critically dependent on the correct balance of dNTP (11). **Figure 2** shows the progressive imbalance in hepatocyte dNTP levels with time on a diet deficient in folate and methyl groups (12,13). The most pronounced alteration was an increase in the ratio of dUTP to dTTP (dUTP/dTTP) consistent with insufficient 5,10-methyl-THF for the methylation of dUMP to dTMP. We and others showed that increased dUTP/dTTP can delay replication fork progression (14) and cell cycle progression (15) and promote genomic instability (16,17). An increase in dUTP/dTTP in vitro was shown to promote folate fragile site expression (18), DNA strand breakage (19), error-prone DNA repair (20), and mutagenesis (21,22). Recent reports of uracil in DNA of folate-deficient human lymphocytes (23,24) confirm earlier reports of uracil misincorporation associated with increased intracellular dUTP/dTTP (25). Using the folate- and methyl-deficient (folate/methyl-deficient) rat model, we show in **Figure 3** that increased uracil misincorporation is preceded by an increase in DNA strand breaks and abasic sites in preneoplastic liver (13). Taken together, these results suggest that an imbalance in dNTP pools (specifically in dUTP/dTTP) can promote the generation of DNA strand breaks and abasic sites that may be related to the reiterative repair of misincorporated uracil in DNA (20).

DNA methyltransferase activity

In mammals, nonrandom cytosine methylation patterns are established in early embryogenesis and are stably inherited during normal cell ontogeny (26). The specific pattern of methylation appears to provide differential accessibility to the DNA information code by affecting DNA-protein interactions and chromatin condensation (27–29). The activity of the maintenance DNA methyltransferase (dnmt1) at the replication fork insures the fidelity and heritability of methylation patterns in normal cells. Shortly after DNA replication, the DNA-bound dnmt1 flips the nascent cytosine base out of the hemimethylated DNA helix and transfers the methyl group from

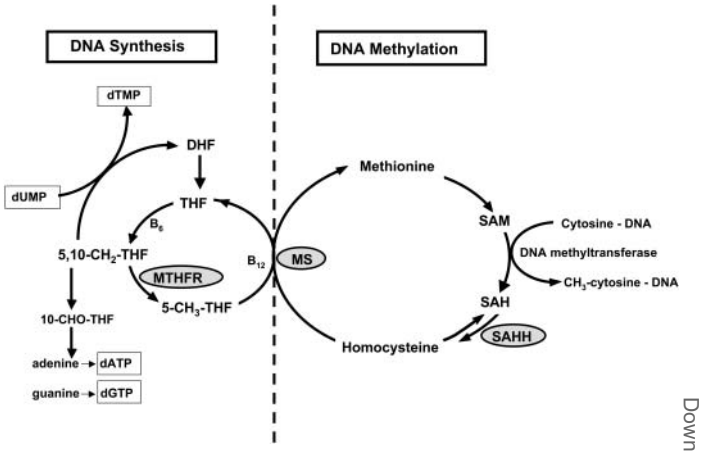


FIGURE 1 The metabolic interactions underlying the importance of folate 1-carbon groups for both DNA synthesis and DNA methylation. The methionine synthase reaction is central for the provision of metabolic precursors for DNA methylation (methionine and SAM) and DNA synthesis (THF and 5,10-CH_2 -THF). Abbreviations: THF, tetrahydrofolate; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; SAM, *S*-adenosylmethionine; SAH, *S*-adensylhomocysteine; DHF, dihydrofolate; dUMP, deooxyuridine monophosphate; dTMP, thymidine monophosphate; dATP, deoxyadenosine triphosphate; dGTP, deoxyguasonosine triphosphosphate.

SAM to the 5 position of the cytosine ring (30). Methylation at hemimethylated CpG sites is referred to as maintenance methyltransferase activity whereas methylation of CpG sites that are totally unmethylated on both strands is referred to as de novo methyltransferase activity. Because the highly conserved dnmt1 also recognizes and binds with high affinity to DNA lesions such as abasic sites, base mispairs, uracil, and other unusual DNA conformations (31,32), it has been proposed that the enzyme evolved from an ancient DNA repair enzyme (33). This possibility is supported by the fact that several other DNA repair enzymes also involve extrahelical base flipping into the active site, including uracil DNA glycosylase, O^6-methyltransferase, *Escherichia coli* photolyase, and exonuclease III (33).

Methylation dysregulation during multistage carcinogenesis

Dysregulation and disruption of the heritable patterns of DNA methylation is a consistent early observation in human

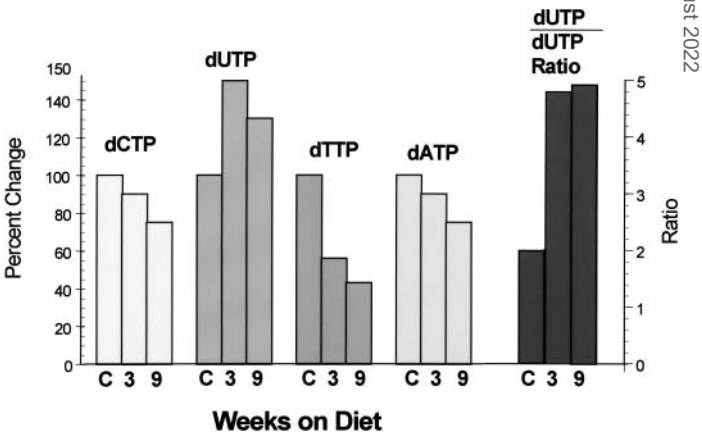


FIGURE 2 The percentage change in hepatic intracellular deoxynucleotide pools in folate/methyl-deficient and control-fed rats after 3 and 9 wk on the folate/methyl-deficient diet (12). *$p < 0.01$.

[3] Abbreviations used: dnmt1, DNA methyltransferase 1; dNTP, deoxynucleoside triphosphates; SAM, *S*-adenosylmethionine; THF, tetrahydrofolate.

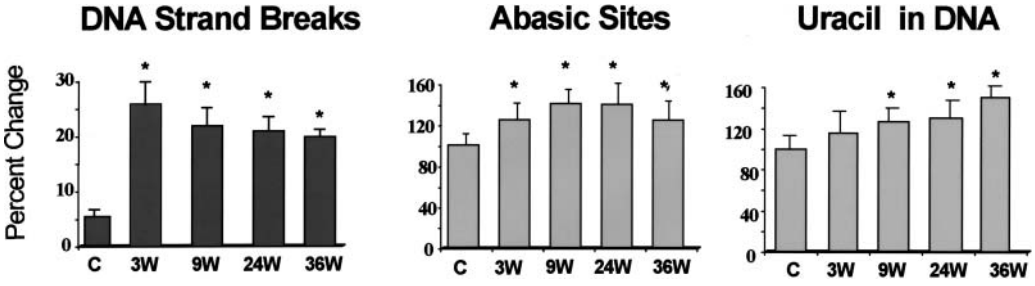


FIGURE 3 The percentage increase in DNA strand breaks, abasic sites, and uracil in DNA in liver of folate/methyl-deficient and control rats after 3,9, 24, and 36 wk of dietary intervention (13,16). *$p < 0.01$.

and experimental cancers (34,35). In general, genome-wide hypomethylation is accompanied by a paradoxical increase in dnmt1 activity and regional hypermethylation in CpG islands of tumor suppressor genes (34). The mechanistic basis for these observations is unclear. Although the intracellular regulation of DNA methyltransferases is poorly understood, chemical or mitogen-induced DNA hypomethylation was shown to stimulate an increase in dnmt1 mRNA (36–38). A major gap in the understanding of methylation dysregulation and neoplastic transformation is a lack of knowledge regarding specific alterations in CpG methylation during early preneoplasia that may be mechanistically related to neoplastic transformation. Present knowledge is based primarily on comparing DNA methylation patterns of normal cells and tumor cells. CpG islands occur within the promoter region of ~60% of human genes and are characterized by increased CpG content relative to the rest of the genome. In normal cells, the CpG dinucleotides are generally methylated within coding regions of most genes but remain remarkably unmethylated in promoter regions (39). Several recent reports have indicated that de novo methylation within CpG islands is associated with transcriptional inactivation of several tumor suppressor genes in human cancers (40–42). Thus, promoter region hypermethylation has recently emerged as a significant alternative to coding region mutation for tumor suppressor gene inactivation. It is not clear whether de novo methylation of promoter CpG islands in tumor cells represents an isolated determining event or a progressive dysregulation of DNA methyltransferase activity. Our methyl-deficient rat model provides a unique opportunity to follow early and progressive changes in DNA damage and DNA methylation in vivo.

The methylation paradox

One of the earliest and most consistently observed alterations in the genome during human carcinogenesis is global hypomethylation of the DNA (43). Paradoxically, the expression of the maintenance methyltransferase dnmt1 and the embryonically expressed de novo methyltransferases dnmt3a and 3b4 have been reported to be increased in preneoplastic liver despite persistent genome-wide hypomethylation (34,44, 45). This paradoxical increase in DNA methyltransferase activity and expression associated with progressive DNA hypomethylation is also observed in the folate/methyl-deficient liver and is shown in **Figure 4**. These observations strongly suggest that despite increased activity and expression, the methyltransferase is unable to maintain normal DNA methylation patterns in the preneoplastic microenvironment. Despite major advances in the understanding of DNA methylation dysregulation and cancer, three fundamental questions remain unanswered. First, what mechanism induces the global loss of methyl groups from DNA during tumor progression? Second, why does the DNA remain hypomethylated despite the increase in DNA methyltransferase activity? Third, what is the signal for de novo methylation in promoter regions of tumor suppressor cells? The elucidation of the mechanisms underlying these questions will provide new insights into the mechanisms by which dysregulated methylation contributes to the carcinogenic process.

DNA methyltransferase and DNA damage

Two independent laboratories studying two different bacterial methyltransferases have demonstrated that the methyltransferase binds to DNA lesions such as uracil, abasic sites, and G:T and G:U mispairs with greater affinity than to its cognate hemimethylated CpG site (31,46). Supporting the preferential binding of the DNA methyltransferase to unusual DNA conformations, Smith et al. (47,48) demonstrated that cytosines (5′ to a normally paired G) opposite mispairs, abasic sites, or gaps within synthetic oligonucleotides were preferential targets for human DNMT1. De novo methylation was also shown to occur preferentially at sites of mispaired cytosines within the spontaneous hairpin loops formed by CGG triplet repeats in the 5′ untranslated region of the human fragile-X

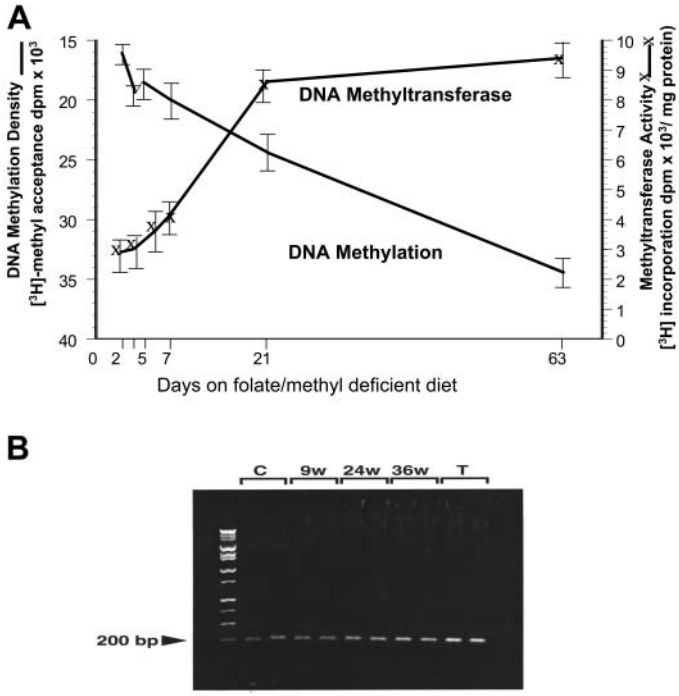


FIGURE 4 (*A*) The progressive decrease in hepatic DNA methylation density and increase in DNA methyltransferase activity after 9 wk on the folate/methyl-deficient diet (16). (*B*) Progressive increase in DNA methyltransferase mRNA levels after 9, 24, and 36 wk on the folate/methyl-deficient diet and in resulting tumors.

FMR-1 gene (49,50). These authors speculate that the presence of a mismatch at the target CpG site creates abnormal base pair stacking interactions that mimic the transition state analog for the methyltransferase and that the enzyme stalls after methyl transfer, forming a stable complex with the conformationally unusual DNA. The preferential binding at cytosine mispairs may also reflect the lower energy requirement for extrahelical base rotation by the enzyme at these sites. In other studies using purified bacterial DNA methyltransferases, replacement of the target cytosine in synthetic oligonucleotides by a mispair, abasic site, uracil, or a gap similarly created binding sites with higher affinity for the methyltransferase than the cognate hemimethylated CpG sites (31,32).

Although rare in the normal cell, high-affinity methyltransferase binding sites (base mispairs, abasic sites, gaps, and strand breaks) are often present as chronic unrepaired DNA lesions in the premalignant cell. We showed that the early appearance and chronic presence of these same lesions in the folate/methyl-deficient model of hepatocarcinogenesis (12,13, 16,51). Sites of DNA damage could bind and preoccupy available methyltransferase such that maintenance DNA methyltransferase activity at the replication fork becomes limiting whereas sites of high-affinity binding may have increased potential for de novo methylation. A deficiency in DNA repair capacity with folate and methyl deficiency would be expected to increase the frequency of ectopic methyltransferase binding at sites of DNA damage and promote DNA hypomethylation at the replication fork.

Resolution of the methylation paradox: hypothesis and evidence

On the basis of these considerations, we hypothesized that genome-wide hypomethylation in hepatic preneoplastic cells and tumors may reflect the high-affinity binding of the DNA methyltransferase to sites of DNA damage such that the maintenance methylation at the replication fork is compromised and the genome becomes progressively hypomethylated with each round of replication. We further propose that when DNA lesions occur within the promoter region of cancer-promoting genes, such as the tumor suppressor genes *p53* and *p16* or DNA repair genes, the aberrant high-affinity binding of the methyltransferase to these lesions is associated with ectopic cytosine methylation; transcriptional repression; and, ultimately, tumor promotion (52,53). An indirect consequence of the site-specific binding of the methyltransferase to DNA lesions would be reduced accessibility of the DNA repair complex leading to reduced DNA repair efficiency and the sustained presence of these lesions. Thus, our hypothesis is compatible with the functional evolution of the enzyme as an ancient DNA repair enzyme and also with the chronic presence of unrepaired DNA lesions that accompany the preneoplastic state of most cancers. This hypothesis for methylation instability associated with carcinogenesis is based on assumption that DNA lesions are a necessary prerequisite for DNA hypomethylation and that hypomethylation without DNA damage may not be carcinogenic. The proposed sequence of events is diagrammed in **Figure 5** and is consistent with the methyl-deficient model of hepatocarcinogenesis and also with preneoplastic DNA damage and the multifactorial progression of multistage carcinogenesis in general.

To test our hypothesis that genome-wide hypomethylation may be an indirect consequence of long-lived methyltransferase-DNA complexes bound to sites of DNA damage, we examined the functional consequences of strand breaks, abasic sites, and uracil in DNA from the folate/methyl-deficient rats on the ability of SssI methyltransferase to transfer methyl groups using the methyl acceptance assay (54). For this experiment, DNA from the deficient rats was divided into two aliquots: one aliquot contained the diet-induced DNA lesions and the second aliquot was rendered lesion-free by in vitro DNA repair treatment. **Figure 6***A* shows the efficiency of the in vitro DNA repair in removing DNA strand breaks, abasic sites, and uracil in DNA from the folate/methyl-deficient rats and that the level of lesions was not different from those present in control DNA. Importantly, both aliquots from the deficient rats were equally hypomethylated and differed only in the level of DNA lesions. To detect potential sequestration of available methyltransferase at sites of DNA lesions, it was necessary to use limiting enzyme concentrations in the methyl acceptance assay. Therefore, we exposed the DNA templates with and without lesions to limiting dilutions of purified SssI methyl-

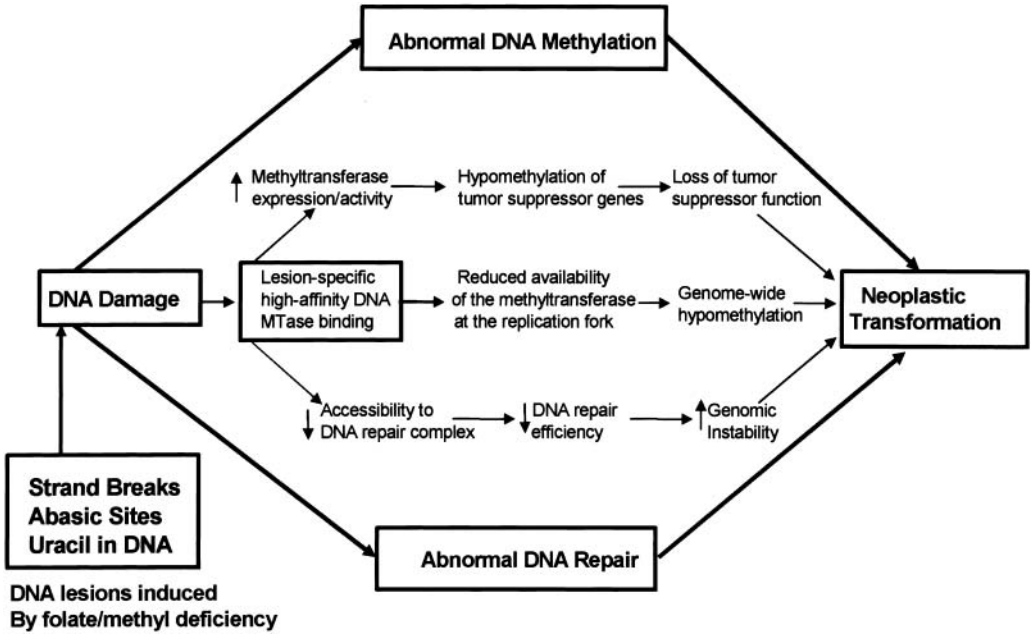
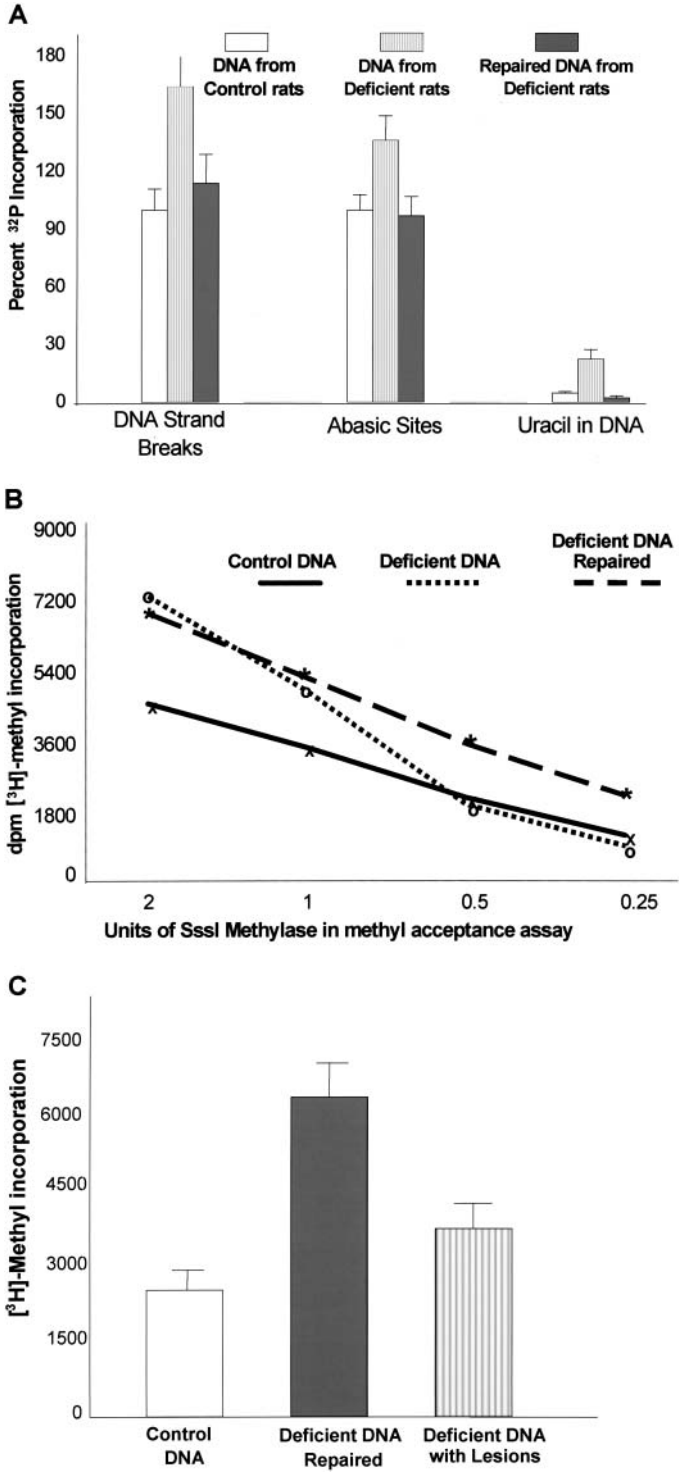


FIGURE 5 An integrated hypothesis that links sites of DNA damage to aberrant high-affinity binding of DNA methyltransferase (dnmt1) and reduced availability of dnmt1 at the replication fork and sites of DNA repair. It is proposed that aberrant binding of the methyltransferase to sites of DNA lesions is mechanistically associated with genome-wide hypomethylation, site-specific de novo methylation, and inactivation of tumor suppressor genes that predispose to neoplastic transformation.

transferase to determine whether the presence of the DNA lesions would reduce the availability of the enzyme to methylate hypomethylated CpG sites. Global DNA hypomethylation is often assessed using the methyl acceptance assay in which the bacterial SssI methyltransferase transfers radiolabeled methyl groups to all unmethylated cytosines in DNA. Thus, the extent of radiolabel incorporation will increase in proportion to the number of unmethylated sites in DNA. Figure 6*B* shows that the repaired DNA from the deficient rats was more hypomethylated than DNA from the control-fed rats (i.e., was capable of accepting more radiolabeled methyl groups at all concentrations of the methyltransferase) (dashed versus solid lines). However, at low enzyme concentrations, the methyltransferase was less able to efficiently transfer methyl groups to the deficient DNA-containing lesions compared with the deficient-repaired DNA (dotted versus dashed lines). Thus, the presence of DNA lesions diminished the ability of the methyltransferase to efficiently methylate the DNA template relative to the repaired DNA template without lesions despite an identical number of hypomethylated sites (Fig. 6C). Although indirect, these observations are consistent with the possibility that the high-affinity binding of the methyltransferase to sites of DNA damage reduced enzyme availability for the transfer of radiolabeled methyl groups to all available hypomethylated sites.



Temporal relationship between DNA damage and DNA hypomethylation

If the loss of cytosine methyl groups during preneoplasia is due, in part, to DNA methyltransferase sequestration at sites of DNA damage, we would predict that genotoxicity should precede DNA hypomethylation. The temporal aspects of DNA damage and DNA hypomethylation are best examined using an in vitro cell model. To this end, we cultured human peripheral blood lymphocytes in folate-deficient medium for 3–7 d and measured the appearance of DNA strand breaks, abasic sites, and global DNA hypomethylation (55). The lymphocytes were purified from whole blood using standard Ficoll-Hypaque centrifugation and cultured in custom prepared–folate deficient RPMI media or control RPMI with phytohemagglutinin at 2 μg/μL, 10% fetal bovine serum, and 1% penicillin/streptomycin. On day three of culture, 10% (by volume) of recombinant interkeukin-2 was added to sustain proliferation; the cells were harvested after 1, 3, 5, and 7 d of culture in folate-deficient or control medium. The data in **Figure 7***A–C* confirm that DNA strand breaks (Fig. 7*A*) precede the appearance of abasic sites (Fig. 7*B*) and that both DNA lesions precede detectable DNA hypomethylation (Fig 7C) using the sensitive cytosine extension assay (56). These results are compatible with in vivo observations in the folate/methyl-deficient rat liver (Fig. 3) and support the premise that DNA damage may be mechanistically related to DNA hypomethylation.

Genome-wide hypomethylation despite increased methyltransferase activity: hypothesis and evidence

The fact that DNA hypomethylation persists despite increased DNA methyltransferase activity and expression

FIGURE 6 (*A*) Relative levels of hepatic DNA strand breaks, abasic sites, and uracil in DNA from control-fed rats compared with DNA isolated from folate/methyl-deficient rats. Half of the DNA from the folate/methyl-deficient livers was exposed to in vitro DNA repair such that the repaired level of lesions was not different from that in the control DNA (open versus solid bars). In contrast, the level of lesions in DNA from the deficient livers without DNA repair (striped bars) was significantly elevated. (*B*) Hepatic DNA from control and folate/methyl-deficient rats with and without in vitro DNA repair were exposed to limiting dilutions of SssI methyltransferase in the methyl acceptance assay. Although the level of hypomethylation was identical between repaired and unrepaired DNA from the deficient rats, the methyltransferase was able to transfer more methyl groups to the repaired DNA than to the DNA containing lesions. (*C*) The ability of the methyltransferase to transfer methyl groups to DNA with lesions was significantly less than that from DNA containing fewer lesions (striped versus solid bars) in the methyl acceptance assay.

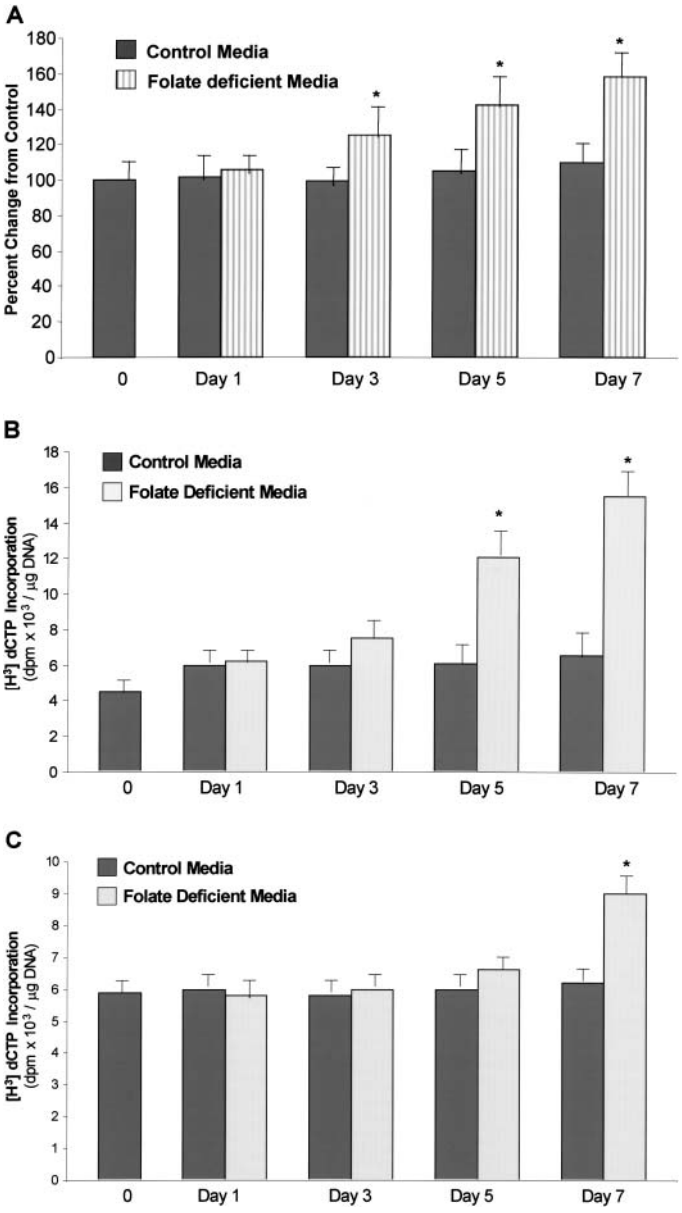


FIGURE 7 The temporal appearance of DNA strand breaks (*A*), abasic sites (*B*), and DNA hypomethylation (*C*) in lymphocytes cultured in control and folate-deficient media for 7 d.

(Fig. 4) indicates that the enzymes are not able to reestablish the normal methylation patterns or density (45,57). We hypothesized that the loss of methylation on both strands of DNA would create heritable CpG sites that would not be recognized by dnmt1, the most abundant DNA methyltransferase in somatic cells (58). A decrease in hemimethylated sites, the preferred substrate for dnmt1, and an increase in double-stranded hypomethylated CpG sites may thereby contribute to the persistence of genome-wide hypomethylation observed in most human and rodent preneoplastic and tumor tissues. To examine this possibility, we determined the progression of hemimethylated CpG sites and double-strand unmethylated CpG sites in DNA from our folate/methyl-deficient rat model.

To measure the extent of hemimethylated sites during preneoplasia, we devised a novel modification of the methyl acceptance assay by using human recombinant DNMT1 rather than the SssI methyltransferase. Unlike SssI, human DNMT1 will preferentially methylate only the hemimethylated sites; therefore, radiolabel incorporation will primarily reflect the level of global hemimethylation. The extent of double-stranded loss of cytosine methylation was measured using the cytosine extension assay after exposure to HpaII, a restriction endonuclease that only cleaves CpG sites that are unmethylated on both strands (56). Thus, by taking advantage of the unique specificities of the two enzymes, we were able to follow the temporal changes in global hemimethylation as well as the accumulation of double-stranded hypomethylated CpG sites in the same DNA samples. Using this approach, we show in **Figure 8***A* that there is a significant increase in hemimethylated CpG sites in DNA from the deficient rats after 3 wk on the deficient diet that is not maintained after 9–36 wk of deficiency. In contrast, Figure 8*B* indicates that the percentage of double-stranded CpG sites increases progressively with time on the deficient diet. These data suggest that CpG methylation is initially lost on one strand and that at later stages of preneoplasia, most CpG sites become unmethylated on both strands of DNA. Given the strong preference of dnmt1 for hemimethylated DNA, it is possible that the predominantly double-stranded unmethylated DNA is simply not recognized by dnmt1 despite the significant increase in enzyme expression and activity (Fig. 4). This possibility would partially explain the paradoxical persistence of DNA hypomethylation in the presence of high methyltransferase activity.

Folate repletion of deficient cells: a double-edged sword

The final question explored in this series of experiments was whether the extensive loss of cytosine methyl groups that

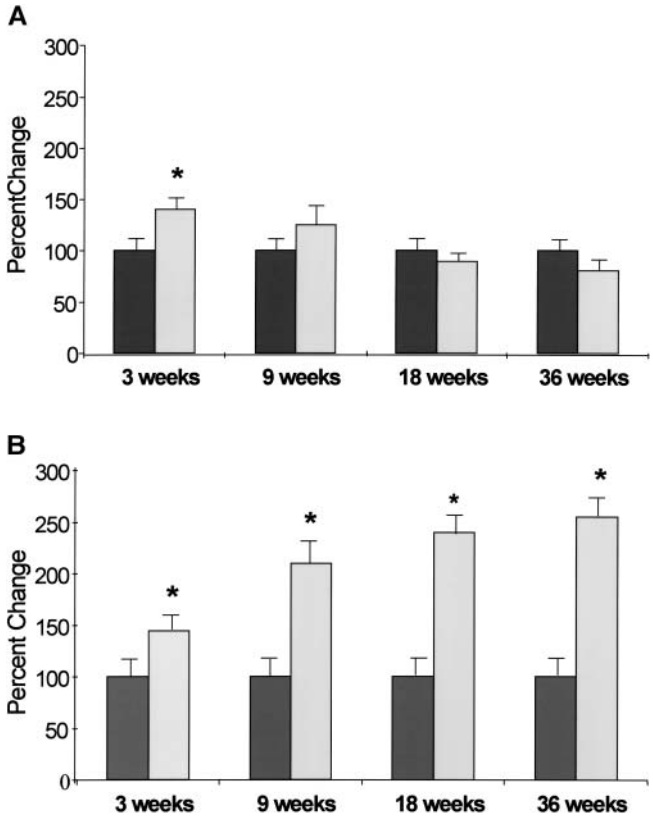


FIGURE 8 (*A*) Significant level of hemimethylated CpG sites in liver of rats fed the folate/methyl-deficient diet for 3 wk that was not different from control levels after 9, 24, or 36 wk of deficiency. (*B*) Progressive increase in double-stranded unmethylated CpG sites in liver of folate/methyl-deficient rats relative to that in control-fed rats.

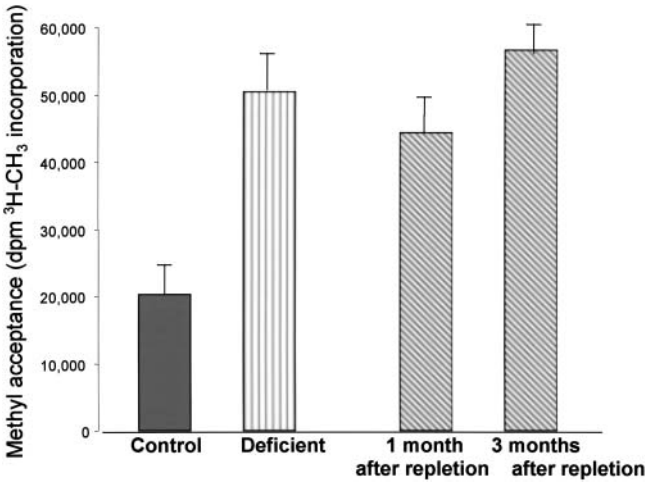


FIGURE 9 Level of DNA hypomethylation in hepatic DNA after 36 wk on the folate/methyl-deficient diet compared with control. DNA hypomethylation was not significantly reversed after 1 and 3 mo of nutritional repletion.

occurs during the extended preneoplastic stage could be reversed and restored to the original methylation density if sufficient methyl groups were provided in the diet. This is a particularly interesting question given the persistent increase in DNA methyltransferase activity. Repletion of methyl donors was accomplished by feeding the control diet to the deficient rats after 36 wk of folate and methyl deficiency (59). The results in **Figure 9** show the significant increase in genome-wide DNA hypomethylation in the deficient liver after 36 wk of deficiency compared with control-fed rats and also show that the level of DNA hypomethylation is not reversed after 1 or 3 mo of repletion with control levels of methionine, choline, and folate. These data indicate that neither the increase in methyltransferase activity nor the increased availability of methyl donors can reestablish the original methylation density in liver of folate/methyl-deficient rats. These data further support our hypothesis that a mechanism underlying the persistence of global DNA hypomethylation may be due in part to the loss of hemimethylated recognition sites for dnmt1, the major DNA methyltransferase in adult somatic cells.

In 1988 Chandar and Lombardi (60) demonstrated that dietary repletion of the methyl donors choline and methionine after 12 mo of a low methionine–choline deficient diet accelerated liver tumor development compared with rats maintained on chronic choline-methionine–deficient diets without repletion. To examine the mechanistic basis for this phenomenon, we cultured DNA repair-deficient CHO-UV5 cells in Ham's F-12 medium or in custom-prepared Ham's F-12 medium lacking in folic acid, thymidine, and hypoxanthine for 18 d without cell passage (61). The results indicated that progressive folate depletion resulted in a significant increase in dUTP/dTTP and the misincorporation of uracil into DNA. These alterations were accompanied by growth inhibition, DNA strand breaks, abasic sites, and phenotypic abnormalities. Acute folate repletion of the deficient cells was used as a proliferative stimulus under conditions of dNTP pool imbalance and multiple DNA lesions. Acute repletion resulted in gene amplification, anchorage-independent growth, and neoplastic cell transformation as evidenced by aggressive tumor growth in Balb/c nu/nu mice (61).

It is generally accepted that metabolic and molecular alterations in cultured cell lines may not be directly applicable to cells in vivo. Nonetheless, experimental control of cells in culture permits a level of manipulation not possible in the multicellular environment in vivo and can provide insights into potential metabolic responses under controlled biochemical stress. Previous clinical and experimental studies have linked nutritional folic acid status to both anticarcinogenic and procarcinogenic activity (62) and suggest that manipulation of intracellular folate can be a double-edged sword depending on the pathophysiologic state of the cell. Although folate supplementation of normal cells appears to be protective, folate supplementation of hypomethylated severely folate-deficient preneoplastic cells containing multiple DNA lesions may promote aberrant DNA synthesis and neoplastic progression (60,61,63,64). The potential negative consequences of acute high-dose folate supplementation to preexisting deficient cells containing DNA lesions warrants some concern. Initial intervention with low doses of folic acid to allow DNA repair without stimulating proliferation may be a prudent alternative to acute high-dose supplementation in individuals with chronic folate deficiency.

Although the evidence presented is based primarily on the folate/methyl-deficient rat model of hepatocarcinogenesis or in vitro folate deprivation, similar DNA lesions and alterations in DNA methylation are often reported in human adenomas and carcinomas. Whether a mechanistic relationship exists between DNA damage and DNA hypomethylation in human cancers as proposed will depend on future research inquiry.

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