

Folate and DNA Methylation: A Review of Molecular Mechanisms and the Evidence for Folate's Role^{1,2}

Krista S. Crider,^{3*} Thomas P. Yang,⁴ Robert J Berry,³ and Lynn B. Bailey⁵

³National Center on Birth Defects and Developmental Disabilities, Division of Birth Defects and Developmental Disabilities, Atlanta, GA,

⁴Department of Biochemistry and Molecular Biology, Center for Epigenetics, Division of Pediatric Genetics, University Florida College of Medicine, Gainesville, FL, University of Florida, Gainesville, FL, and ⁵Foods and Nutrition Department, University of Georgia, Athens, GA

ABSTRACT

DNA methylation is an epigenetic modification critical to normal genome regulation and development. The vitamin folate is a key source of the one carbon group used to methylate DNA. Because normal mammalian development is dependent on DNA methylation, there is enormous interest in assessing the potential for changes in folate intake to modulate DNA methylation both as a biomarker for folate status and as a mechanistic link to developmental disorders and chronic diseases including cancer. This review highlights the role of DNA methylation in normal genome function, how it can be altered, and the evidence of the role of folate/folic acid in these processes. *Adv. Nutr.* 3: 21–38, 2012.

Introduction

Folate is an essential water-soluble vitamin occurring naturally in select foods as well as in the synthetic form (folic acid) used in supplements and in food fortification programs (1–3). There are many critical cellular pathways dependent on folate as a 1-carbon source including DNA, RNA, and protein methylation as well as DNA synthesis and maintenance. Folate can be a limiting factor in all these reactions (Fig. 1).

Epigenetics is the study of heritable changes in phenotype or gene expression that do not result from changes in the primary DNA sequence (4,5). Recognized mechanisms of epigenetic regulation in mammals include DNA methylation, post-translational modification of histones, chromatin remodeling, microRNAs, and long noncoding RNAs (6,7). These epigenetic regulatory mechanisms modulate chromatin structure and contribute to regulation of the major molecular processes in the nucleus including transcription, replication, repair, and RNA processing. DNA methylation

is a covalent modification of genomic DNA that modifies gene expression and provides a mechanism for transmitting and perpetuating epigenetic information through DNA replication and cell division. The role of DNA methylation in cellular regulation has also provided the potential for a new paradigm of disease intervention and treatment. The development of various inhibitors of DNA methylation that alter methylation patterns within intact mammalian cells has led to the clinical use of some inhibitors in experimental therapies for human diseases such as hematological malignancies (8) and myelodysplastic disorders (9).

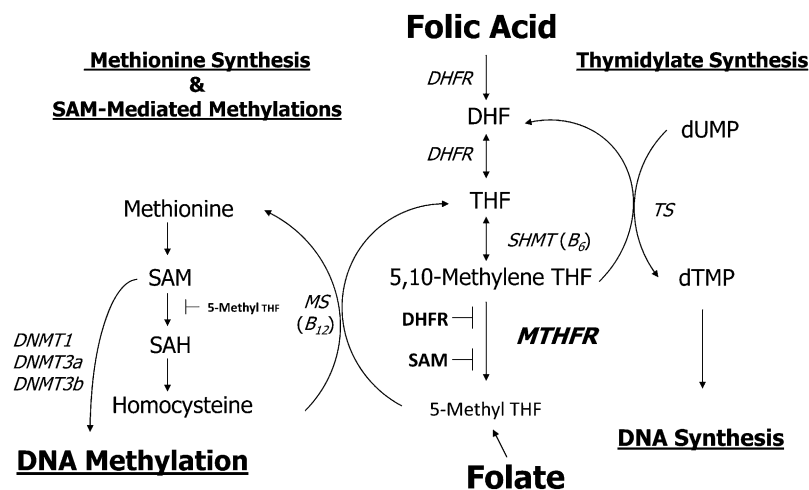
DNA methylation patterns are stable and are retained in purified genomic DNA; therefore, studies of DNA methylation are amenable to a wide variety of cell-free assays and technologies including DNA methylation analysis at single-nucleotide resolution, next-generation sequencing, and genome-wide methylation profiling (10) (Table 1). Currently, new DNA sequencing technologies are beginning to provide novel insight into genome-wide patterns of DNA methylation (10–13). Although high-resolution truly genome-wide studies have been limited to a very small sample size, a recent study described the DNA methylation level at 1505 individual sites (loci) in 808 genes in 1628 human genomic DNA samples (14). Even this tremendous data set only covers a tiny portion of the genome in a limited number of samples. Despite these recent advances, a basic understanding of normal variation in genomic

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² Supplemental Table 1 and Supplemental Figure 1 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at <http://advances.nutrition.org>.

* To whom correspondence should be addressed. E-mail: kcrider@cdc.gov.

Figure 1 Folic acid metabolism. This schematic shows the process by which folate/folic acid is used for DNA methylation. The *MTHFR* 677C→T variant reduces enzyme activity (175) and may help to divert the available methyl groups from the DNA methylation pathway toward the DNA synthesis pathway (176–178). The pathway is complex and highly regulated, with feedback loops and interactions not shown in the schematic. Gene names for enzymes are in italics and cofactors are in parentheses. DHF, dihydrofolate; DHFR, dihydrofolate reductase; DNMT, DNA methyltransferase; dTMP, thymidylate; dUMP, deoxyuridine monophosphate; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate; TS, thymidylate synthase.



patterns of DNA methylation in humans across tissues, age, populations, disease, or environmental conditions (including dietary intakes) have not been well described. The methods needed to undertake these types of studies and the infrastructure to do these analyses are rapidly emerging (10,15,16).

This review was written to provide the nutritional scientist with a synopsis of the mechanistic aspects of DNA methylation as a background for understanding the potential for the nutrient folate to affect these same molecular processes. Specifically, highlights of the following are covered: the roles and mechanisms of DNA methylation and demethylation; the mechanisms of gene silencing by DNA methylation; the function of DNA methylation; reprogramming of DNA methylation patterns during development and differentiation; and the importance of changes in DNA methylation (hyper- and hypomethylation). Following this, the potential role of folate in the DNA methylation process and its assessment as a folate status biomarker and link to disease outcomes are covered: folate's role in 1-carbon metabolism related to DNA methylation; low folate status and DNA methylation; background of DNA methylation, cancer, and folate; studies of cancer patients and folate status and global DNA methylation; studies of healthy adults and folate status; changes in DNA methylation in response to the environment and diet—the importance of the developmental timing of exposure; studies of folate in the fetus, infants, cord blood; and high folate and folic acid intake and DNA methylation. The review concludes with a focus on research issues including methodological considerations that are key in planning and interpreting research investigations related to assessing differences in DNA methylation in response to changes in folate status. Clearly there are many challenging research issues that need to be addressed to fill in the gaps in our knowledge related to the potential for folate to modulate DNA methylation and potentially have an impact on development and health maintenance.

Current status of knowledge

Part I: overview of DNA methylation

Introduction to DNA methylation. Methylation of cytosine is common throughout the human genome. This covalent modification most commonly occurs at cytosines within a 5'-CpG-3' dinucleotide when a methyl group from S-adenosylmethionine (SAM)⁶ is enzymatically transferred to the 5 position of cytosine to generate 5-methylcytosine (5-MC) in genomic DNA. DNA methylation patterns are a product of the frequency of cytosine DNA methylation at specific sites along a strand of DNA. Recent genome-wide high-resolution DNA methylation analysis of a primary human fibroblast cell line demonstrated that 4.25% of total cytosines in genomic DNA are methylated, 67.7% of CpGs are methylated, and 99.98% of DNA methylation occurs in CpG dinucleotides (10). Similar analysis of a human embryonic stem cell line showed that 5.83% of cytosines are methylated, 82.7% of CpG dinucleotides are methylated, and 25% of all cytosine methylation occurs at non-CpG sites (10); an earlier study also reported high levels of non-CpG methylation in mouse embryonic stem cells in contrast to methylation patterns in mouse somatic cells (17). Although the importance of CpG methylation is established, the role of non-CpG methylation is a new active area of research.

Interestingly, the frequency of CpG dinucleotides is lower than would be expected throughout most mammalian genomes (18). This is believed to be due to the spontaneous deamination of 5-MC to yield thymine and a cytosine-to-thymidine transition in DNA (19). However, certain regions of the genome have approximately a 10-fold higher

⁶ Abbreviations used: BER, base excision repair; BPA, bisphenol A; DMR, differentially methylated region; DNMT, DNA methyltransferase; 5-HMC, 5-hydroxymethylcytosine; IAP, intracisternal A particle; 5-MC, 5-methylcytosine; 5-methylTHF, 5-methyltetrahydrofolate; LINE, long interspersed element; MTHFR, methylenetetrahydrofolate reductase; NTD, neural tube defect; RBC, red blood cell; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SINE, short interspersed element; THF, tetrahydrofolate.

Table 1. Representative list of commonly used methodologies for analyzing DNA methylation¹

| | Description | Pros | Cons | References |
|--|---|--|---|--|
| Global | | | | |
| Liquid chromatography tandem mass spectrometry | Digestion/hydrolysis of genomic DNA to nucleosides and fractionation by LC-MS | Highly sensitive, quantitative, accurate, and reproducible | Requires costly instrumentation; requires $\sim 1 \mu\text{g}$ amounts of DNA | Shellnut et al. (137) |
| HPLC | Digestion/hydrolysis of genomic DNA to nucleotides or bases and fractionation on HPLC | Highly quantitative, accurate, and reproducible | Requires costly instrumentation; requires μg amounts of DNA | Armstrong et al. (186) |
| Methyl acceptance | Global methylation determined by level of in vitro methylation of genomic DNA by SssI methylase | Technical simplicity | Lacks sensitivity; imprecise; weak reproducibility | Balaghi and Wagner (187) |
| Gene specific | | | | |
| Methyl-sensitive restriction enzyme digestion | Assesses methylation at specific restriction sites using Southern blotting | Standard molecular biology techniques | Limited to analysis of individual restriction sites; not strictly quantitative | Wolf and Migeon (188) |
| Methylation-specific polymerase chain reaction | Specifically amplifies methylated genomic DNA sequences after sodium bisulfite treatment of genomic DNA | Sensitivity; technical simplicity | Not quantitative; limited sequence coverage | Herman et al. (189) |
| Pyrosequencing | Determines level of methylation at specific CpG sites | Highly quantitative; single-nucleotide resolution; technically straightforward | Requires costly instrumentation; restricted sequence coverage | Tost and Gut (190) |
| Microarray | Large numbers of individual CpG sites from specific genes are assayed using standard microarray equipment after bisulfite treatment; 1500 > 450K sites per sample | Quantitative; specific; technically straightforward; large number of CpGs at a time | Requires costly instrumentation; requires $\sim 0.5 \mu\text{g}$ DNA | Bibikova et al. (191), Christensen et al. (131), Fernandez et al. (14) |
| Sodium bisulfite sequencing | Determines methylation status of each cytosine in a region of interest within a single DNA molecule | Single-nucleotide resolution; reveals DNA methylation patterns of individual DNA molecules | Labor intensive; time-consuming; restricted sequence coverage | Clark et al. (192) |
| Genome-wide profiling | | | | |
| MethylC-seq | Determines methylation status of each cytosine in the genome after chemical conversion of unmethylated cytosines to uracils in genomic DNA | Single-nucleotide resolution; full genome coverage; the most comprehensive method for genome-wide profiling of DNA methylation | Requires sequencing of full genome at ~ 15 -fold coverage; cost | Lister et al. (45) |
| meDIP/meDNA pull-down/MIRA | Uses antimethylated cytosine antibody or methylated DNA-binding protein to enrich for methylated DNA | Successfully used in a wide variety of published studies; technically accessible | Low resolution, not full genome coverage; efficiency dependent on CpG density | Weber et al. (193), Nair et al. (194), Rauch et al. (195) |
| RRBS | A size-selected portion of the genome is isolated after restriction enzyme digestion and sequenced after sodium bisulfite treatment | Single-nucleotide resolution | Biased toward analyzing CpG islands; lacks full genome coverage | Gu et al. (196) |
| CHARM | Uses restriction enzyme that specifically cleaves methylated DNA | Quantitative | Lacks full genome coverage | Irizarry et al. (197) |
| HELP | Compares the relative representation of MspI and HpaII fragments at individual loci | Assays both CpG islands and non-CpG island sites; quantitative | Lacks full genome coverage | Khulan et al. (198) |
| MSSC | Assesses methylation status at all recognition sites of a methylation-sensitive restriction enzyme | High resolution; not restricted to CpG islands | Not strictly quantitative; indirect assessment of methylation by detecting unmethylated CpG sites; lacks full genome coverage | Ball et al. (199) |

¹ For sodium bisulfite conversion used in methylation-specific polymerase chain reaction, pyrosequencing, sodium bisulfite genomic sequencing, full genome sequencing, full genome sequencing in genomic DNA are chemically converted uracil by sodium bisulfite; methylated cytosines are resistant to conversion and can be detected by various methods. (This table is not a comprehensive list of methods used to assay DNA methylation) CHARM, comprehensive high-throughput arrays for relative methylation; HELP, HpaII tiny fragment enrichment by ligation-mediated polymerase chain reaction; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; meDIP, methylated DNA immunoprecipitation; meDNA, methylated DNA; MIRA, methylated CpG island recovery assay; MSSC, methylation-sensitive cut counting; RRBS, reduced representation bisulfite sequencing.

frequency of the CpG dinucleotide than the rest of the genome. These regions are referred to as CpG islands and are often correlated with the location of genes, particularly promoter regions and other regulatory regions (20,21). These islands compose <1% of the genome and are typically unmethylated (Fig. 2). Mapping CpG islands has been used as a tool for gene discovery because >50% of all mammalian genes are associated with CpG islands (19,22–24). CpG islands were originally defined as genomic regions ~200 base pairs in size with a C+G content of 50% and an observed CpG/expected CpG >0.6 (20), although this definition is somewhat arbitrary and has been refined more recently to correlate more closely with promoter regions and regulatory regions (21) (Fig. 2). Another functionally

important region for DNA methylation are CpG island “shores,” which are CpG-containing regions that are within 2 kb of a CpG island, contain low densities of CpG sites, and have been shown in recent work to be a location of tissue-specific differential methylation in normal tissues (13). Methylation of CpG island shores has been correlated with gene expression, and certain shores have shown altered methylation patterns in colon cancer (13).

DNA methylation and demethylation: roles and mechanisms. Methylation at CpG dinucleotides provides a mechanism for transmitting DNA methylation patterns after DNA replication and perpetuating patterns of epigenetic regulation through subsequent cell generations. Methylation

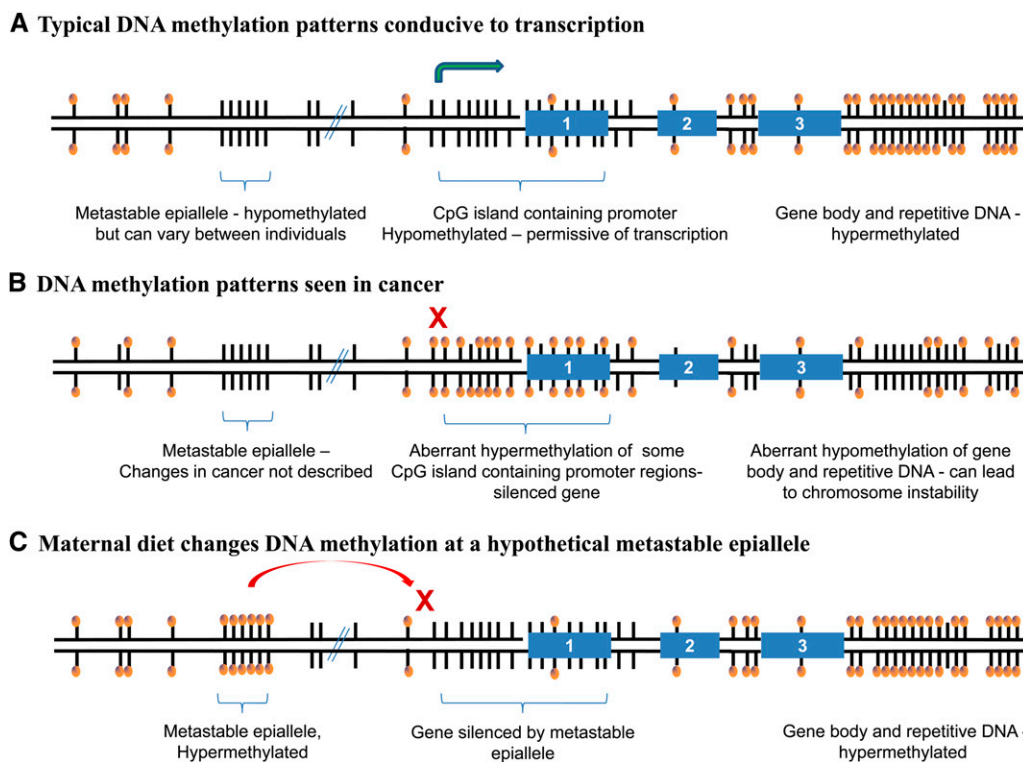


Figure 2 DNA methylation patterns at different types of genetic loci and how the patterns change in cancer and in response to maternal diet. This is a cartoon of a hypothetical single section of double-stranded DNA. Each bar represents a 5'-CpG -3' site. Methylated CpG are indicated by bars with balls at the end. The green arrow indicates transcription start and active transcription. The red X equals gene silencing. The numbered blue rectangles are exons. (A) DNA methylation patterns conducive to transcription:– patterns set during development. CpGs throughout the genome are targeted for methylation by DNA methyltransferases (DNMT1, DNMT3a, DNMT3b). CpG islands are regions with a concentration of CpGs often (but not always) associated with the promoter regions of genes. CpG islands generally are thought to be actively protected from DNA methylation to allow for appropriate regulation of transcription (179). (B) Dysregulation of DNA methylation seen in cancer post-transformation. Cancer cells have aberrant DNA methylation patterns. Overall DNA methylation levels are reduced (58,180–182). A subset of genetic sites is hypermethylated, including some tumor suppressor genes; the specific loci hypermethylated vary among types of tumors (114,131). (C) Maternal diet changes methylation levels at the metastable epiallele, which can change transcription of a neighboring region. Metastable epialleles are regions of the genome that have a variable methylation patterns that are semihheritable across generations, illustrated by the *agouti* mouse model (147). In mice, a maternal diet that includes additional methyl donors can lead to increased methylation at the metastable epiallele (the intracisternal A particle retrotransposon), a change in expression at nearby genetic loci, and permanent changes in the phenotype distribution of the offspring (147). The sequence of the retrotransposon is a candidate for methylation in the early embryo; however, the extent to which it becomes methylated varies in genetically identical individuals based on maternal diet (methyl donors as well as other dietary factors) (146,183). A recent study found candidate loci in the human genome that show differential methylation dependent on season of birth (148).

of CpG dinucleotides results in symmetrically methylated CpG sites (i.e., methylation of the CpG sequence on both strands of DNA) (as seen in Fig. 2). However, after DNA replication, the 2 resulting daughter DNA molecules become hemimethylated with the parental template strand maintaining its methylation and the newly synthesized strand lacking methylation. Continued DNA replication of these hemimethylated daughter molecules would eventually result in the loss of DNA methylation patterns in subsequent generations. In 1975, Riggs (25) and Holliday and Pugh (26) proposed a scheme by which DNA methylation patterns at CpG dinucleotides could be restored in newly synthesized DNA after DNA replication. They postulated that hemimethylated CpG sites generated by DNA replication could be specifically recognized by a maintenance DNA methyltransferase that would methylate CpG sites on the newly synthesized DNA strand immediately after DNA replication. This would regenerate the patterns of symmetrically methylated CpG dinucleotides in daughter cells and maintain the epigenetic states and patterns of gene regulation that existed in parental cells. In 1983, a DNA methyltransferase (DNMT) (now referred to as DNMT1) was identified that demonstrated the predicted preference for hemimethylated DNA (27,28). Thus, the general mechanism is now widely accepted as a mechanism for maintaining DNA methylation patterns after DNA replication and cell division, although more recent evidence suggests that maintenance methylation alone may not be sufficient to fully perpetuate DNA methylation patterns genome-wide (29). Nonetheless, maintenance DNA methylation of hemimethylated substrates provides a clear example of a mechanism that allows the transmission and retention of epigenetic information through multiple cell generations. It has been postulated that the availability of folate as a source of methyl groups may affect the ability to maintain DNA methylation patterns in replicating cells (30,31).

Mammals have 3 types of DNMT: DNMT1, DNMT3a, and DNMT3b (32). DNMT1 is the most abundant DNMT in cells and, as previously described, is believed to act as the primary maintenance methyltransferase to methylate hemimethylated DNA after DNA replication and preserve parental DNA methylation patterns in daughter cells. In contrast, DNMT3a and DNMT3b function as de novo methyltransferases to methylate fully unmethylated CpG sites. They function primarily during mammalian development to establish DNA methylation patterns as epigenetic remodeling and reprogramming proceed during differentiation. DNMT3a and DNMT3b methylate different subsets of DNA sequences in the genome as evidenced by the different phenotypes of mice carrying a knockout of either methyltransferase (32). These de novo methyltransferases also appear to play a role in maintenance methylation to fully preserve parental DNA methylation patterns in daughter cells (29).

Although DNA methylation has usually been regarded as a relatively stable epigenetic mark, there are clear examples where DNA demethylation in mammalian cells occurs in the absence of DNA replication and cell division, suggesting

the existence of mechanisms of active DNA demethylation (33). However, studies of DNA demethylation have been controversial and mechanisms of demethylation in mammals still are not well understood (33). One mechanism of passive DNA demethylation involves inhibition or loss of maintenance methylation at CpG sites so that multiple rounds of DNA replication and cell division eventually result in the loss of DNA methylation at these sites in subsequent cell generations. Several mechanisms of active DNA demethylation also have been postulated and reported, but none have been conclusively demonstrated. One potential mechanism of active demethylation involves direct removal of the methyl group via cleavage of a highly stable C-C bond; however, reports demonstrating this mechanism have not been subsequently confirmed or reproduced (34–36). Other potential mechanisms of active demethylation involve base excision repair (BER) of DNA after events such as removal of 5-MC bases in DNA by glycosylase activity or enzymatic deamination of 5-MC to thymidine and formation of a T:G mismatch; in either case, the excised base would be replaced with an unmethylated cytosine by BER (37). Nucleotide excision repair has also been reported to play a role in active DNA demethylation, although other studies seem to contradict this mechanism (38–40). Most recently, the appreciation that notable levels of 5-hydroxymethylcytosine (5-HMC) are present in mammalian genomic DNA and that conversion of 5-MC to 5-HMC in DNA can be catalyzed by mammalian TET (ten-eleven translocation) proteins has generated interest in the possible role of 5-HMC in mechanisms of DNA demethylation (41–43). One potential mechanism of 5-HMC-mediated demethylation would involve recognition and removal of 5-HMC and replacement with an unmethylated cytosine by BER. Thus, what was once regarded as a highly stable and terminal epigenetic state, DNA methylation is becoming increasingly viewed as a more dynamic process. As more genome-wide methylation studies are reported, there is evidence of both increases and decreases in DNA methylation on differentiation (44–46). Furthermore, the finding of variation in DNA methylation between the sexes and changes in methylation during aging suggests that there is more interindividual variation and variation in DNA methylation patterns than was previously expected (47–50).

Mechanisms of gene silencing by DNA methylation.

Methylation of CpG islands, especially those islands colocalized with promoters or other regulatory regions, is generally associated with gene repression. The mechanisms by which DNA methylation silences transcription are not fully understood, although several mechanisms have been proposed. First, methylation of a specific regulatory DNA sequence and the attendant insertion of the methyl group into the major groove of DNA may prevent stable binding of a regulatory protein (e.g., transcription factor) to that sequence, thereby directly preventing gene activation by a transcription factor. In vitro binding studies have identified sequence-specific DNA-binding transcription factors whose

interaction with their cognate DNA recognition sequence is negatively affected by methylation of a CpG dinucleotide within the binding sequence (51). However, many transcription factors are not sensitive to DNA methylation and not all DNA sequences bound by a sequence-specific transcription factor will contain a CpG dinucleotide. Therefore, other more indirect mechanisms must also be involved in repressing transcription by DNA methylation. One such indirect mechanism is via DNA-binding proteins containing methylated DNA-binding domains (52). These methylated DNA-binding proteins, such as methyl CpG-binding protein 2 and methylated DNA-binding domain 2, recognize and bind to 1 or more methylated CpG dinucleotides. These proteins in turn interact with or recruit transcriptional silencing complexes containing chromatin remodeling complexes and/or histone modification enzymes (e.g., histone deacetylases) that act to form a transcriptionally repressive condensed chromatin structure at the associated gene (53–55). DNMTs have also been found as components of multisubunit chromatin modifying and remodeling complexes involved in transcriptional repression, suggesting that the DNA methylation machinery also may be recruited to specific genes by other mechanisms (similar to those that recruit histone modification and remodeling activities) and that chromatin modification/remodeling and DNA methylation act cooperatively to silence transcription. In contrast, intragenic DNA methylation of active genes is often higher than in promoter regions, suggesting that methylation in the body of genes is a positive regulator of gene expression; a recent study suggests that this may occur by suppressing the repressive effects of the polycomb complex, which is associated with chromatin remodeling (56).

Function of DNA methylation. Cytosine methylation has been hypothesized to be an ancient component of the immune system designed to recognize and inactivate parasitic viral DNA sequences that infiltrate the genome (57). Mammalian genomes contain a large proportion of repetitive DNA sequences (e.g., nearly 50% of human genomic DNA) that include retroviral sequences, transposons, retrotransposons long interspersed elements (LINEs), short interspersed elements (SINEs), extended blocks of tandemly repeated DNA sequences (e.g., satellite DNA), etc. Most of the DNA methylation present in mammalian genomes is associated with these repetitive DNA sequences and serves to suppress the potential activity and deleterious effects of repetitive DNA (58–60). This includes suppressing mobility and propagation of repetitive elements capable of transposition and suppressing illegitimate recombination between related repetitive elements (58–60). These functions serve to prevent widespread genome instability and rearrangement mediated by repetitive DNA sequences (61).

It is hypothesized that over time the function of DNA methylation has evolved and now plays additional roles, including transcriptional regulation, X-chromosome inactivation, imprinting, and tissue-specific gene expression (19,62,63). Hypermethylation of CpG islands is highly

correlated with transcriptional silencing of the associated gene, particularly for CpG islands associated with a gene promoter region (55). One clear example of this is the aberrant stable transcriptional silencing of certain tumor suppressor genes in cancer cells by the hypermethylation of their promoter CpG islands (64). However, methylation of a CpG island does not necessarily lead to gene silencing. For example, the gene for telomerase has been shown to be activated by methylation (65). DNA methylation also occurs within the body of genes and between genes; the function of methylation at CpG sites in these regions still is poorly understood. Also, it is not clear whether methylation of critical CpG sites within CpG islands triggers hypermethylation of the island and gene silencing (66), whether overall levels of hypermethylation within a CpG island govern silencing, or whether both mechanisms contribute to silencing. Recently, in contrast to CpG island methylation, reports indicate that methylation in the body of genes has a role as a positive regulator of transcription (56) and may also have a role in transcription elongation (67), alternative promoter use (68), and nucleosome positioning (69). Generally, DNA methylation is thought to maintain gene silencing versus initiating gene silencing (70) such that a gene is silenced initially by another mechanism and that silencing is stabilized by methylation of associated regulatory regions (e.g., CpG island, promoter region).

X-chromosome inactivation is the process by which female mammals compensate for the unequal dose of X-linked genes between male (XY) and female (XX) cells (71). Females randomly inactivate 1 of the 2 X chromosomes present in each and every somatic cell in a process that occurs in early female embryogenesis, resulting in monoallelic expression of genes subject to X-chromosome inactivation. Once a female cell in early embryogenesis randomly chooses which X chromosome to inactivate (i.e., the maternally or paternally inherited X chromosome), all mitotic progeny of that cell maintain inactivation of the same X chromosome throughout the remainder of development into adult tissues. One of the hallmarks of the inactive X chromosome is the hypermethylation of promoter CpG islands along the length of the inactive X, which contributes to the stable maintenance of transcriptional silencing of the associated genes on the inactive X chromosome (72); these same CpG islands are typically unmethylated on the active X chromosome.

Genomic imprinting is another mammalian epigenetic system of monoallelic gene expression in which the 2 alleles of a particular gene within the same cell are differentially regulated, in this case, in a parent-of-origin fashion (73). Thus, for some imprinted genes, only the paternally inherited allele is expressed, whereas the maternally inherited allele is stably silenced. Conversely, for other imprinted genes, the paternally inherited allele is stably silenced and only the maternally inherited allele is transcriptionally active. This differential monoallelic expression of the maternal or paternal allele is usually associated with differential DNA methylation of 1 or more regulatory regions of these genes. These

so-called differentially methylated regions (DMRs) are generally (but not always) hypermethylated on the transcriptionally silenced allele and hypomethylated on the expressed allele. Imprinting control regions that regulate imprinting across an entire domain of imprinted genes are typically DMRs (74,75). The differential parent-of-origin DNA methylation patterns at imprinted genes are established during gametogenesis where DNA of the male and female gametes acquire either maternal- or paternal-specific methylation patterns before fertilization (although some parent-of-origin methylation patterns may also continue to develop post-zygotically) (Supplemental Fig. 1A) (76). These differential gametic methylation imprints are then maintained and propagated during subsequent somatic development to facilitate the parent-of-origin patterns of monoallelic transcription associated with imprinted genes. Imprinted genes are thought to comprise a small proportion of mammalian genomes with <100 currently confirmed in humans and mice (77). In humans there is a limited number of known imprinted loci (<80), and only a subset of these are shared with the mouse (78,79). However, a recent study of imprinting in embryonic and adult mouse brains found parent-of-origin bias in the expression of 1300 genes and transcript isoforms and, if confirmed, would suggest that imprinting (or parent-of-origin effects) may be more widespread and complex than originally believed (80).

Reprogramming of DNA methylation patterns during development and differentiation. DNA methylation patterns undergo remodeling during the course of mammalian differentiation and development to eventually generate the cell type-specific methylation patterns found in adult somatic cells. Dynamic changes in methylation are particularly pronounced during gametogenesis and early embryogenesis (Supplemental Fig. 1). During the course of gametogenesis, DNA methylation patterns are erased (i.e., demethylated) within each developing germ cell (primordial germ cells) and then remethylated and reset to the genomic DNA methylation patterns specific to either the sperm or the egg as gametogenesis proceeds (81,82). This process is particularly important for establishing the gamete-specific methylation patterns of imprinted genes in sperm and egg before fertilization. Current data suggest that other nonimprinted loci may also be subject to at least partial erasure, and resetting of DNA methylation patterns during gametogenesis (83–85). In early embryogenesis, DNA methylation patterns also undergo a genome-wide process of erasure, that in mouse embryogenesis begins shortly after fertilization and continues to the blastocyst stage (86). This wave of demethylation erases the methylation patterns of the gametes and is followed by DNA remethylation during the remaining course of embryogenesis to establish the genome-wide and cell-type specific DNA methylation patterns found in fully differentiated adult somatic cells (44,87,88). In small studies of human embryos, it has been shown that there is a wave of demethylation at the 4-cell stage followed by remethylation beginning as early as the late morula (89). An important

exception to this dynamic reprogramming of DNA methylation patterns during embryogenesis occurs with imprinted genes that escape this process of erasure and resetting, thereby maintaining throughout embryogenesis the differential DNA methylation of the paternally and maternally inherited alleles in somatic cells characteristic of most imprinted loci (90).

These periods of dynamic reprogramming of DNA methylation patterns during gametogenesis and embryogenesis may also present windows of opportunity for environmental influences (e.g., diet and nutrition) on the developing embryo to alter the normal process of establishing DNA methylation (and other epigenetic) patterns (91–93). Such changes in DNA methylation patterns could affect normal patterns of gene expression or alter genome stability, thereby leading to an increased risk of diseases later in life, even if these changes in methylation occur in only a subset of cells of a given tissue.

It is also possible to alter DNA methylation levels and patterns within intact mammalian cells by treatment with various chemical inhibitors, most commonly cytidine analogs such 5-azadeoxycytidine. 5-Azadeoxycytidine (and its ribonucleotide analog 5-azacytidine) functions as a demethylating agent (9), resulting in hypomethylation of the genome in treated cells. These cytidine analogs and other DNA-demethylating drugs have recently been introduced as potential therapeutic agents for the treatment of human diseases, particularly myelodysplastic syndromes (9).

What does a change in DNA methylation reflect? Hypermethylation versus hypomethylation. Any CpG site in a single DNA molecule can either be methylated or unmethylated, so the level of methylation at that site will either be 0% or 100%. However, within a single diploid cell, the methylation level of a given CpG site on 1 strand of DNA can be 0% (symmetrically unmethylated on both chromosomes), 50% (symmetrically methylated on 1 chromosome and unmethylated on the other chromosome, as often seen in DMRs of imprinted genes), or 100% (symmetrically methylated on both chromosomes). DNA methylation assays are usually performed on a population of cells; therefore, the level of methylation at a given CpG site in these assays reflects the collective methylation at this site in multiple cells (and multiple DNA molecules) and the variation in methylation seen at individual CpG sites. Thus, a finding that a particular CpG site is 50% methylated in a population of cells could mean that this site is 50% methylated in every cell (i.e., 1 allele methylated and the other allele unmethylated), that this site is unmethylated in 50% of the cells in the population and fully methylated in the other 50% of cells, or some other combination of methylation levels among cells in the population that yields a composite methylation level of 50%. The different functional consequences of these 3 different scenarios are important.

When considering the implications of changes in the percentage of methylation at a specific CpG site (e.g., in response to dietary conditions), it is important to consider

that a 10% change in DNA methylation level at a site could be distributed across an entire population of cells (and not appreciably affect transcription), reflect a 100% change in a subpopulation of cells (and represent gene silencing or activation in these cells), or something in between. Each of these potential scenarios has different implications for the impact of the same 10% change in methylation level.

In the case of CpG islands, gene silencing is generally associated with multiple methylated CpG sites across a strand of DNA (Fig. 2). However, site-specific methylation can result in gene silencing if the methylated CpG blocks the binding of a DNA methylation-sensitive transcription factor. At most genetic loci, it is unknown which CpG sites or how many along the strand of DNA must be methylated to silence any given gene. When evaluating the impact of changes in DNA methylation, it is important to consider the exact location assayed and the potential of methylation at that site or region to change gene expression or other biological processes of interest.

Part II: Folate and DNA methylation

Folate's role in 1-carbon metabolism related to DNA methylation. Under normal dietary conditions, absorbed folate is metabolized to 5-methyltetrahydrofolate (5-methylTHF, monoglutamyl form) in the intestine and/or liver. 5-MethylTHF is the primary folate constituent taken up by nonhepatic tissues, which then must be polyglutamated for cellular retention and 1 carbon cycle coenzyme function. Tetrahydrofolate (THF) is the most effective substrate for polyglutamate synthetase; therefore, 5-methylTHF must be converted to THF via the methionine synthase reaction (Fig. 1). When folic acid is consumed in fortified foods or supplements, it is metabolized primarily to 5-methylTHF during intestinal absorption and/or first pass in the liver, after which it behaves identically to natural dietary folate. Folic acid is normally first reduced to dihydrofolate by dihydrofolate reductase and subsequently to THF to enter the folate pool (Fig. 1). In some cases, the capacity of dihydrofolate reductase is exceeded and folic acid may appear in the circulation in the oxidized form (94). Once the THF coenzyme is formed from either folic acid or dietary folate, it is first converted to 5,10-methyleneTHF by the vitamin B-6-dependent enzyme serine hydroxymethyltransferase and subsequently irreversibly reduced to 5-methylTHF by methylenetetrahydrofolate reductase (MTHFR). This reaction is key to maintaining the flux of methyl groups for the remethylation of homocysteine to methionine via the vitamin B-12-dependent methionine synthase reaction. Methionine is the substrate for SAM or AdoMet, a cofactor and methyl group donor for numerous methylation reactions including the methylation of DNA, RNA, neurotransmitters, and other small molecules, phospholipids, and proteins, including histones (95). A number of SAM-dependent reactions have regulatory roles by affecting both genome stability and gene transcription (55), localization of protein (96), and small molecule degradation (97).

In addition to folate, a number of other dietary nutrients are required to maintain 1 carbon flux, ensuring normal

homocysteine remethylation, SAM formation, and DNA methylation. These nutrients include vitamin B-6 (serine hydroxymethyltransferase activity), riboflavin (MTHFR stability), vitamin B-12 (methionine synthase function), and choline (betaine precursor as a hepatic methyl source via betaine:homocysteine methyltransferase) (98).

The 1-carbon pathway, and thus DNA methylation, functions under tight regulatory controls. SAM is the major regulator of folate-dependent homocysteine remethylation because it is a potent inhibitor of MTHFR. Under the condition of high SAM concentration, MTHFR is inhibited, which reduces the synthesis of 5-methylTHF and hence remethylation of homocysteine. Conversely, when SAM concentrations are low, remethylation of homocysteine is favored. MTHFR activity and thus 5-methylTHF formation may also be modified by the common genetic variant, 677C → T, which reduces enzyme activity (99). It is also recognized that S-adenosylhomocysteine (SAH) functions as a potent product inhibitor of SAM-dependent methyltransferases (100). For this reason, continual hydrolysis of SAH to homocysteine is essential to maintain normal DNA methylation (101). Moderate elevations in plasma homocysteine concentration have been reported to be associated with increased concentration of SAH, but not SAM, and increased SAH concentration has been associated with global DNA hypomethylation (102).

Low folate status and DNA methylation. Low folate status (as defined by various measures including blood folate concentrations, folate intake, and/or folic acid intake) has been associated with an increased risk of cardiovascular disease, multiple cancers, and neural tube defects (103–105). The mechanisms by which low folate status contributes to these disorders remain unclear. During DNA replication, folate depletion can have destabilizing consequences. Inadequate folate availability during cell division can result in the compromised production of thymidine, such that uracil may be substituted in the DNA sequence (Fig. 1). This mutagenic event may trigger attempts to repair the defect and increases the frequency of chromosomal breaks (104). In tissue culture, it has been shown that low folic acid results in the formation of micronuclei (indicative of chromosome breakage) and that the *MTHFR* TT genotype leads to increased micronuclei formation under low folate conditions (106).

DNA methylation, cancer, and folate. Global hypomethylation and targeted hypermethylation are considered defining characteristics of human tumors (14,23,58,107,108) (Fig. 2B). DNA methylation patterns are widely dysregulated in human cancer (Fig. 2B). Early in the study of DNA methylation, genome-wide hypomethylation was found in tumor tissues compared with matched healthy tissues (58). Additionally, ~5% of genes were found to be hypermethylated in nonrandom patterns specific to the type of tumor (109). It was determined that these changes in DNA methylation in tumors resulted in silencing of tumor suppressor genes and chromosome instability (Fig. 2B) (110,111).

Hypomethylation of repetitive elements may be predictive of increased risk of cancer and increased mortality from cancer (112). In recent large-scale DNA methylation studies of multiple types of primary tumors (thousands of loci in hundreds of tumors), DNA methylation changes in tumors were associated with concomitant changes in gene expression and tumor characteristics (113,114). The DNA methylation changes in cancer do not appear to be random because tumors of specific cell lineages show similar DNA methylation changes that are distinct from other tumor types and normal tissues. As tumors increase in severity, a progressive number of loci show increased DNA methylation primarily at CpG islands and decreased DNA methylation in non-CpG islands sites (14). The magnitude of DNA methylation changes observed in cancer tissues is substantially greater than any differences in DNA methylation between types of normal tissue (14).

Folic acid intake has been reported to prevent loss of heterozygosity of a tumor suppressor gene in a small supplementation trial in humans (115). Folate depletion of human tissue culture cells can result not only in the expected global hypomethylation (116,117) but also targeted hypermethylation of the H-cadherin locus (117). It is unclear whether increases in dietary folate and/or folic acid result in changes in healthy tissues that can predispose to carcinogenesis. Many studies of the effects of folic acid supplementation have focused on either preventing or promoting cancer, especially colon cancer (**Supplemental Table 1**).

Studies of cancer patients and folate status and global DNA methylation. There has been great interest in reversing epigenetic changes seen in early tumorigenesis (e.g., global hypomethylation) as rapidly dividing tissue tumors may be susceptible to low folate availability, resulting in global hypomethylation. Three clinical trials examined the impact of folic acid supplementation (0.4–10 mg/d) on global DNA methylation in colon cancer patients (30,118,119) (**Supplemental Table 1**). These studies have shown that DNA methylation might be a biomarker for colorectal cancer and that methylation of DNA in the colon and leukocytes can be increased by supplementation with folic acid in human patients (118,120). Among the observational studies (e.g., cohort and case-control) of the association of folate status (measured as folate/folic acid intake and or blood folate concentration) and cancer, 5 studies examined the global DNA methylation level (121–125) (**Supplemental Table 1**). In these studies, an association was found between cancer and global DNA hypomethylation in circulating blood and/or tumor tissue; however, there was not a consistent association between global DNA methylation and folate status (intake or blood folate) across studies (121–125). This lack of a definitive association between low folate status and global DNA hypomethylation could be due to a limited sample size or imprecision of the assays. Across studies outlined in **Supplemental Table 1**, global hypomethylation was found in tumors and even normal colon cells and blood of cancer patients (121,122,124,126).

The association of global hypomethylation with folate status (e.g., intake, blood folate concentration) has been inconsistent among studies of cancer patients (**Supplemental Table 1**). This is not unexpected given the variety of study designs, different global DNA methylation assays, and types of tissues examined. There are many techniques available to assess “global” DNA methylation level including those that look at repetitive element methylation level LINE, SINE, and Alu and those that examine various restriction sites throughout the genome. However, the correlation between these measures is actually quite low (127). This is not necessarily due to the accuracy of the measure but due to the fact that although they can be thought of as global, they are actually measuring different types and subsets of DNA sequences in disparate regions of the genome that may be regulated differently. Indeed, recent work suggests that there are different changes in DNA methylation between types of tumors and types of repetitive DNA elements often used as proxies for global methylation (128). Additionally, each of the early trials of global DNA methylation in colon cancer patients used the methyl acceptance assay (30,118,119). This assay provides an estimate of global DNA methylation by using an enzyme to methylate purified genomic DNA with radiolabeled tritium. Methyl acceptance is limited in precision and necessitates intact DNA (129) so that the use of damaged DNA (which may occur as a result of the outcome of interest or during preparation or handling) will result in inaccurate results. Careful consideration of assay methodology is critical for study design to ensure appropriate interpretation and reproducibility of the data.

Studies of cancer patients and folate status and site-specific DNA methylation. For patients with a previous adenoma of the colon, to date, only 1 trial examined the effect of folic acid supplementation (1 mg/d for 3 y) on site-specific DNA methylation, and it was limited to 2 loci (49). In tumors, methylation of tumor suppressor CpG islands is generally either fully methylated and silenced (>80% methylated), or unmethylated (<20% methylated) depending on the exact location of the CpG sites (130). The changes in DNA methylation observed in association with variation in red blood cell (RBC) folate concentration in humans are much smaller than those present in tumors. Wallace et al. (49) evaluated DNA methylation levels at 2 CpG island-containing genes in colon tissue of persons with a previous adenoma after a placebo-controlled folic acid supplementation trial (1 mg/d for 3 y). They found a trend of greater methylation at each genetic locus as the quintile of RBC folate increased [*ERα*: lowest RBC folate quintile (Q1 43.3–520.9 μg/L) vs. highest RBC folate quintile (Q5 1081.2–2620.8); 10.3% vs. 11.3% methylated; *P*-trend = 0.03 and *SFRP1*: Q1 vs. Q5; 21.2% methylated vs. 23.0% methylated *P*-trend = 0.01]. Although statistically significant, the functional impact of these small changes in DNA methylation (<2%) on gene transcription or longer term health outcomes is unclear at this time. There was no association of DNA methylation at either of these loci

with treatment with 1 mg/d folic acid for 3 y, plasma folate concentration, or dietary folate intake. Although age, protein intake, and race were strongly associated with DNA methylation at these loci. In this study, there were no statistically significant associations of methylation at either of these loci with hyperplastic polyps or adenomas after 3 y of treatment.

Two additional observational studies of cancer examined site-specific DNA methylation and folate status (131,132). Kim et al. (132) examined DNA methylation at 3 genetic loci and found that high plasma folate concentration among cases ($\geq 6.7 \mu\text{g/L}$ vs. $< 4.1 \mu\text{g/L}$) was associated with an increased methylation at *p73* but not *p16^{in4A}* or *hMLH1*. Christensen et al. (131) recently examined a cohort of women with breast cancer and the DNA methylation level at 1413 sites in 733 genes. They found that dietary folate intake was associated with DNA methylation class membership in primary breast tumors (131). This type of study examining thousands of loci simultaneously will likely produce a better understanding of the interaction between DNA methylation and folate, although substantial analytic and informatics challenges exist.

It is important to note that low folate status can lead to both hypo- and hypermethylation, resulting in misregulation of this complex system. The changes observed in cancer do not appear to be random such that not all CpG islands are susceptible to hypermethylation (133,134). Some of these changes appear to precede transformation (initiation of cancer), and others may be a consequence of transformation. At this time, it is unclear whether subsets of genetic loci are susceptible to DNA methylation changes in response to folate/folic acid intake in cancer patients; the studies highlighted in Supplemental Table 1 show a number of loci that warrant examination in future studies. Large-scale genome-wide analysis may provide additional candidates.

Studies of healthy adults and folate status. In healthy adults, dividing tissues such as the blood and the gut mucosa are likely to be most susceptible to low folate in the diet. In humans, 3 studies examined the effects of controlled folate depletion and repletion on global DNA methylation in leukocytes (135–137) (Supplemental Table 1). Folate depletion resulted in reductions in global DNA methylation in older women in controlled feeding studies (135,136), a finding that was not observed by Shelnutt et al. (137) in younger female adults. Both Jacob et al. (136), and Shelnutt et al. (137) observed considerable increases in methylation with folate intake during the repletion period. In the Shelnutt et al. (137) study, the increases were limited to those with the *MTHFR* TT genotype (see Fig. 1 for location in the folic acid cycle). Axume et al. (138) did not find DNA methylation changes in response to depletion or repletion; however, the study was restricted to those with the *MTHFR* CC genotype. Although the results from the controlled feeding trials of healthy adults are somewhat inconsistent, these studies are limited by small sample size ($n = 8\text{--}33$ individuals per

group), differences in age groups, different initial folate status, and varying lengths of intervention.

Two additional observational studies examined global DNA methylation and folate status in patients without cancer (126,139) (Supplemental Table 1). Friso et al. (139) found an *MTHFR* genotype-dependent association between lower global DNA methylation and lower plasma folate concentration. Pufulete et al. (126) observed a trend toward lower DNA methylation with lower serum folate concentration. Taken together, these findings suggest that there may be a differential global DNA methylation response to folate depletion and repletion dependent on age, genotypes, duration, and magnitude of exposure. None of these studies of healthy adults examined site-specific DNA methylation changes; however, in a study of men with hyperhomocystinemia, global hypomethylation was reversed and monoallelic expression of imprinted genes restored in patients given folic acid therapy (140) (Supplemental Table 1).

Changes in DNA methylation in response to the environment and diet: the importance of the developmental timing of exposure. There is increasing evidence of more interindividual variation and variation in DNA methylation patterns across the life span than was previously expected, and these changes may be modulated by environmental exposures. DNA methylation varies between the sexes and changes during aging (47–50). In addition, an increasing number of environmental exposures in humans such as air pollution (141), benzene exposure (142), and particulate pollution (141) have been linked to changes in DNA methylation (e.g., repetitive element hypomethylation) in adults. Furthermore, Friso et al. (143) found that estrogen replacement resulted in lowering of plasma homocysteine concentration and increases in global methylation in a small ($n = 13$ postmenopausal women) double-blind, placebo-controlled, double-crossover study, whereas no changes in methylation were found at the *ER α* , *ER β* , and *p16* genes. Thus, certain environmental exposures have been associated with changes in DNA methylation and changes in 1-carbon metabolites.

There has been increasing interest in in utero effects of environmental exposures (from toxins to nutrition) on long-term health outcomes, and it has been hypothesized that disease risk may in part be determined by maternal and paternal diet (87,144,145). Studies of cancers have found that there are changes in DNA methylation in the blood that reflect the cancer risk in the tissue of interest (119,124). This may be because dividing tissues are susceptible to the same insults or that the change that resulted in the predisposition to cancer happened in the embryonic period and is reflected in many tissues (Supplemental Fig. 1C).

In mice it is clear that maternal dietary changes in 1-carbon availability can affect the DNA methylation patterns and phenotype of offspring (87). The best-studied example is the *agouti* mouse. The *agouti* mouse strain has an insertion of an intracisternal A particle (IAP) sequence in an upstream

region of the *agouti* gene that encodes a paracrine signaling molecule. The *agouti* gene in this strain is now regulated by the promoter activity of the IAP. Increasing the availability of methyl donors in the mother's diet during pregnancy resulted in increased DNA methylation of the IAP and phenotypic changes associated with altered regulation of the *agouti* gene in offspring (146,148). Recently, Waterland et al. (148) found loci in the human genome that also may act as metastable epialleles (similar to the *agouti* locus in the *agouti* mouse) that show differential methylation in response to season of birth in a small human study. In both of these studies, the identity of the causative agent for changes in DNA methylation is unclear because there were a pool of methyl donors supplied in the *agouti* mouse model, and multiple dietary factors were altered in the small human study.

Epigenetic effects of other dietary components are of interest as well. The offspring of male mice fed a low-protein diet (from weaning to sexual maturity) showed numerous although modest (typically 10–20%) changes in DNA methylation in the liver, with altered expression of genes involved in lipid and cholesterol biosynthesis, compared with control offspring of fathers fed a normal protein diet (149). Paternal high-fat diets were reported to reduce DNA methylation at the *Il13ra2* gene in mouse pancreatic islet cells of female offspring (150). Interestingly, epigenetic effects of certain environmental conditions in utero appear to be abrogated by dietary folic acid supplementation. In rats, a maternal low-protein diet has been reported to increase DNA methylation of the imprinting control region of the imprinted *Igf2/H19* domain in the livers of offspring compared with control offspring whose mothers were fed a normal protein diet. This increase in methylation in response to the low-protein diet was prevented by dietary supplementation of the low-protein diet with folic acid (151). A similar outcome was reported for prenatal exposure to the widespread environmental contaminant bisphenol A (BPA). In the *agouti* mouse strain, changes in DNA methylation at the *agouti* locus in response to BPA exposure in utero (via maternal dietary consumption of BPA during pregnancy) were negated by supplementation of the BPA-containing diet with folic acid (152). Thus, certain environmental conditions and diets of parents may have epigenetic effects on their offspring and dietary folic acid can, in some cases, counteract the epigenetic effects of these environmental conditions on offspring. This would suggest the potential for a complex interplay between the effects of maternal and paternal environments and diets on the epigenome of offspring.

Extreme exposures can be used as proof-of-principle to show plausibility of a cause-and-effect relationship. Studies of prenatal starvation have shown an association with many adverse health outcomes including both short-term and long-term consequences: neural tube defects (NTDs), metabolic syndromes, and increased risk of schizophrenia, depending on the precise timing of the exposure and the sex of the fetus (153–158). The period around conception may be one of the more sensitive periods and corresponds

to the time when the epigenetic patterns are reset (Supplemental Fig. 1B). It has been hypothesized that epigenetic changes as a result of starvation exposure may be responsible for these effects later in life. However, it is not clear which macronutrient or micronutrient deficiency or combination thereof may be responsible for these health outcomes.

Studies in fetus, infants, cord blood, and folate status. As a result of the erasure and resetting of DNA methylation patterns in early development and differentiation as well as the rapid growth rate in early development, early development could be particularly susceptible to folate intake. Two studies showed epigenetic changes in individuals prenatally exposed to famine during the Dutch Hunger Winter at the end of World War II (which might be indicative of extreme folate deficiency) (153,159) (Supplemental Table 1). Heijmans et al. (159) found methylation changes in *IGF2* associated with prenatal exposure to prenatal famine. Tobi et al. (153) examined 15 loci important for growth and metabolic disease and found that methylation was lower at *INSIGF* and higher at *IL10*, *LEP*, *ABCA1*, *GNASAS*, and *MEG3* in persons exposed to famine periconceptionally compared with their sex-matched siblings (all $P < 0.001$). An interaction with sex was found for *INSIGF*, *LEP*, and *GNASAS*. Exposure to famine at later gestational ages resulted only in changes to *GNASAS* ($P < 0.001$). In men only *LEP* was significantly associated with later exposures.

The association between maternal use of folic acid and DNA methylation in offspring was examined in 1 study. Steegers-Theunissen et al. (160) found that maternal use of folic acid was associated with a 4.5% increase in *IGF2* DMR methylation (49.5% vs. 47.4%, $P = 0.014$) in children. *IGF2* DMR methylation in the child was associated with maternal SAM level and inversely with birth weight (−1.7% methylation per SD birth weight, $P = 0.034$). Although highly significant, the absolute changes observed in these studies were a <3% change in the DNA methylation level of loci that are normally unmethylated. It is unknown whether these DNA methylation changes would result in functionally relevant transcriptional changes.

A recent study by Chang et al. (161) compared the DNA methylation level in various tissues from terminated fetuses (18–28 wk) affected by NTDs with matched normal controls. There were differences in the level and patterns of global DNA methylation between the NTD-affected and control fetuses. There was hypomethylation in the brain of fetuses with NTDs compared with controls ($P < 0.01$). In addition to the lower mean serum folate concentration in mothers with NTD-affected pregnancies, a correlation between a woman's serum folate concentration and DNA methylation in the brain tissue of NTD-affected fetuses was found (161) (Supplemental Table 1).

Three recent studies examined the relationship between maternal folic acid supplementation in cord blood on methylation of the *LINE-1* repetitive element (162), site-specific methylation (27,578 genetic loci) (163), *IGF2* promoter methylation (164), and both *IGF2* and *H19* (165)

(Supplemental Table 1). These studies showed no association of folate status (as defined as serum folate concentration or folic acid intake) with global or site-specific DNA methylation (162–164). However, a fourth study by Hoyo et al. (165) found an association between increased folic acid intake and decreased DNA methylation at *H19*. Additionally, there was an association of increasing maternal plasma homocysteine concentration and decreasing percentage of methylation of LINE-1 ($r = -0.688$, $P = 0.001$) (162), and significant correlation between methylation patterns (cluster in the modeling of the site-specific methylation) and plasma homocysteine concentration ($P = 0.038$), LINE-1 methylation ($P = 0.028$), and birth weight percentiles ($P = 0.019$) (163). Additionally, Ba et al. (164) found that maternal and cord blood vitamin B-12 concentrations were associated with P3 (third promoter CpG site) methylation (both $P < 0.01$). P2 was also associated with maternal weight gain and exposure to smoking ($P = 0.03$ and $P = 0.02$).

These studies show the possibility that folate/folic acid in the maternal diet could change DNA methylation levels in the offspring. However, these relationships are complex and can involve other components of the diet and/or substrates from 1-carbon metabolism, suggesting no definitive linear associations of folate/folic acid intake in the mother with increases in DNA methylation in the child.

High folate and folic acid intake and DNA methylation.

The purpose of many of the controlled feeding trials was to attempt to reverse the aberrant DNA methylation observed in cancer. The effect of folate insufficiency is clearly detrimental both to the embryo and its short-term risk of NTDs and the possible longer term risks of diabetes or other health outcomes (156,157,166,167). Additionally, high blood folate concentrations (121) and high global DNA methylation have been shown in a number of studies to reduce the risk of cancer (123,125). However, recent focus has shifted to concern about folic acid supplementation resulting in progression of existing tumors and altering normal DNA methylation patterns (31,168,169). Currently, there is no direct evidence of aberrant DNA methylation and change in gene expression in response to “high” levels of folate/folic acid intake. In addition, there is no consensus on a dose of folic acid or a blood folate concentration that would be associated with potential adverse health outcomes.

Measurement of DNA methylation is not indicative of a single process or phenomenon. Different regions of the genome are differentially regulated and would not be expected to respond similarly to any given exposure. DNA methylation is a highly regulated process dependent on time, tissue, DNA sequence, region of the genome, and a multitude of other regulated enzymes and proteins. Simple correlations, such as high folate status leading to increases in DNA methylation, are unlikely to apply broadly; indeed the conflicting results presented in **Supplemental Table 1** illustrate the complexity of the process.

In the very limited number of studies to date, folic acid supplement use was not associated with the complete

methylation and silencing of CpG island-associated tumor suppressor genes observed in cancer among adults (49) or children (160). Promoter regions of genes are highly regulated and CpG islands in promoters generally remain protected from DNA methylation under normal circumstances. Even in tumors, loci susceptible to hypermethylation vary among tumor types (108,113,114,131,170,171). As previously discussed, a recent study by Hoyo et al. (165) examined the DNA methylation level in the cord blood of newly delivered infants at the *H19* DMR and found significantly decreased DNA methylation level (toward the expected 50% level) with increasing maternal folic acid intake in pregnancy, suggesting to Hoyo et al. that folic acid intake in a fortified population may provide additional benefit. At this time, there are insufficient data to determine whether there is an effect of higher doses of folic acid at any particular locus, genomic region, specific tissue type, or developmental state and whether the change would result in increased risk or benefit.

Part III: Future research

Although several studies have begun to examine the relationship between DNA methylation and folate (**Supplemental Table 1**), many studies are underpowered in terms of the number of participants, tissues types, and number of epigenetic loci examined. None of the findings associating folate status (indicated by folate/folic acid intake or blood folate concentration) with changes in global or site-specific DNA methylation have been consistently replicated. It remains an open question whether folate/folic acid intake is associated with changes in DNA methylation and whether there is an interaction with other micronutrients or genetic variation. Even in experimental animal models that show changes in DNA methylation after increased intake of 1-carbon sources, it is unknown whether the effects are due to folic acid or one of the other 1-carbon sources (e.g., betaine, choline) or a combination thereof (147). Because folate is not the only vitamin in the 1-carbon cycle and DNA methylation is not the only methylation reaction that may affect health, studies designed to evaluate interactions are needed.

As technology enables the greater use of genome-wide analyses of DNA methylation at single nucleotide resolution, the previously held dogma surrounding aspects of DNA methylation and folate status may require modification. No population-based, randomized, controlled trial has examined the effects of folic acid on DNA methylation levels and patterns in healthy humans. In planning new studies, it is important to consider the appropriate assay, tissue to test, type of DNA methylation change anticipated, the folate/folic acid exposure of interest, DNA methylation patterning, and the timing of the folate/folic acid exposure (**Table 2**).

Enumerable questions about the relationship between folate status and DNA methylation need further exploration and include the following: What is the normal variation in DNA methylation level and pattern across the genome and between tissues and developmental stages?

Table 2. Questions to consider when planning or evaluating a study on the effect of folic acid or folate on DNA methylation

| | |
|-----------------------|--|
| Assay | <p>DNA methylation patterns vary across genomic regions. These regions are differentially regulated and the consequences of changes in DNA methylation vary depending on the site and developmental time frame. It is important to consider:</p> <ul style="list-style-type: none"> • Does the assay test individual sites (alone or a multiplex format) or “global” methylation? • Is the assay sensitive and specific? What % change in methylation level can be detected? Is the % change meaningful? Is this known? • Can the assay differentiate between 5 methyl cytosine and 5 hydroxymethylcytosine? Is this of importance to the outcome of interest? • For the assay of choice what is the accuracy, reproducibility, labor intensiveness, and sample usage and cost (both for testing and analysis)? |
| Tissue | <p>DNA methylation patterns vary between tissues at specific loci. It is important to consider:</p> <ul style="list-style-type: none"> • Is the tissue being testing composed of one or more than one cell type? • Is the “normal” methylation pattern and level known for the CpG sites being testing? Is there methylation variation at those sites or regions among tissues/cell lineages? • Is there a known correlation between methylation in the tissue being assaying and the tissue/outcome of interest (e.g., blood vs. tumor or buccal vs. brain)? |
| Type of Change | <p>The “normal” variation in DNA methylation is unknown for many regions of the genome (not just among tissues but in the same tissue between “normal” individuals within the population). It is important to consider:</p> <ul style="list-style-type: none"> • What is the normal methylation pattern at the site(s)/region of interest? Is the normal variation in methylation known? • Is the exposure of interest anticipated to increase or decrease the DNA methylation level? • Are the sites of change known to affect gene expression? |
| Time | <p>Exposures at different developmental times could be expected to have differential impacts.</p> <ul style="list-style-type: none"> • Developmentally, are the DNA methylation patterns of specific interest set in the tissue at the specific time during development? Is it known? • Are the patterns set at the same developmental time in the tissue of interest and the tissue assayed (if different)? • Are both tissues/cells types replicating? |
| Exposure | <p>The mechanisms that lead to aberrant methylation levels in response to lower and higher folate or folic acid intake could vary. It might be anticipated that a deficiency would lead to more issues in replicating cells due to lack of substrate. It is unknown if higher intakes are associated with aberrant changes in DNA methylation.</p> <ul style="list-style-type: none"> • What is the exposure? For how long? At what age? • What are the cut-offs for exposure (folate/folic acid intake, RBC, plasma or serum folate levels) or is it a continuum? • Is the effect modified by other cofactors in the one-carbon pathway? • Is the machinery present to aberrantly methylate DNA (e.g., in the tissue of interest, which DNA methylation transferases are expressed?) • Is it possible that the epigenetic and/or phenotypic effects of exposure (particularly during pregnancy) occur in offspring? • Are epigenetic effects of exposure potentially transgenerational? |

Are there systematic differences in DNA methylation between different populations that vary with micronutrient intake? Are there health consequences due to the small changes in DNA methylation levels/patterns observed in many of the existing studies? How does folate status interact with other nutrients to affect DNA methylation level/patterning and does this vary depending on the developmental timing or tissue assayed? How does DNA methylation vary in relation to timing and dose of folate or specifically folic acid intake?

Conclusions

Appropriate DNA methylation patterns are critical to normal genome function. Aberrant DNA methylation patterns are present in many human diseases, including cancer, imprinting disorders, and developmental disabilities (172–174). The human studies of DNA methylation and folate/folic intake acid vary widely in their study design, timing of exposure, and amount of folate/folic acid intake, tissue tested, assays, and, not surprisingly, the findings. At this time, the evidence suggests that low folate status is

associated with decreases in global DNA methylation, which has in some studies has been associated with an increased risk of cancer. However, it is unclear how specific regions of the genome respond to higher or lower folate intakes. To date, the majority of studies have only examined a limited number of genetic loci and/or a small number of samples. There is no direct evidence that high dietary folate or folic acid intake leads to aberrant DNA methylation, changes in gene expression, or disease state. Given the conflicting results to date, it is clear that the current research does not support a linear relationship or dose response between folic acid supplementation and global or site-specific DNA methylation level. This is not unexpected given that DNA methylation is part of a complex, highly regulated system. Additional research is needed to elucidate the relationship between folate and DNA methylation.

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