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GENETIC EFFECTS OF METHYLATION DIETS

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■ **Abstract** DNA methylation at cytosines in CpG dinucleotides can lead to changes in gene expression and function without altering the primary sequence of the DNA. Methylation can be affected by dietary levels of methyl-donor components, such as folic acid. This may be an important mechanism for environmentally induced changes in gene expression. Recent literature supports a role for DNA-methylation changes in a number of adult-onset disorders and during development. These changes may be significant for better understanding certain birth defects (e.g., neural tube defects) and the long-term consequences of early environmental influences on gene expression (metabolic programming). Optimal "methylation diets" should be investigated as part of the prevention and treatment of all these conditions, as well as in disorders such as Rett syndrome, whose primary defects may lie in DNA methylation-dependent gene regulation.

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INTRODUCTION

Folic acid, vitamin B12, and other nutrients influence the function of enzymes that participate in various methylation processes by affecting the supply of methyl groups that these enzymes incorporate into a wide variety of molecules. Methylation modifies genomic DNA without altering its primary sequence (epigenetic modification), but also contributes to functionally relevant modifications of RNA, phospholipids, proteins, and the synthesis of neurotransmitters. This review focuses on the effects of DNA methylation, which in mammals occurs primarily at cytosine in CpG dinucleotides and has been proposed to play a role in genomic stability and regulation of gene function.

In brief, cytosine methylation in CpG islands within a gene's promoter region correlates with its transcriptional silencing: Methylation correlates with modification of histone proteins in the nucleosomes and compaction of chromatin, which renders it unavailable to transcription factors. The major methyl donor for the various methyltransferase enzymes is S-adenosylmethionine (SAM), which derives from methionine. Methionine in turn is formed when the enzyme methionine synthase (MTR) adds a methyl group from 5-methyltetrahydrofolate (5MTHF) to homocysteine; methionine can also be formed when a methyl group from betaine is added to homocysteine by the enzyme betaine-methionine homocysteine methyl transferase (BHMT). The methylcobalamin form of vitamin B12 is the cofactor of MTR, and zinc is a cofactor of BHMT.

There is evidence from in vivo and in vitro studies, mostly in cancer research, that variation in the dietary levels of folic acid can alter DNA methylation and gene expression. This may contribute to risk for cancer as well as its development and progression. Variations in DNA methylation are associated with aging (77), atherosclerosis (123), and chronic inflammatory disorders (76) and may contribute to variations in gene expression during development (141) and risk for

birth defects (72, 80, 87). The nutrients that play a role in this pathway also influence various cognitive processes, perhaps in part by affecting DNA methylation (127). How diet might affect methylation has been studied in mouse models for folate-responsive neural tube defects (87) and in an interesting model of epigenetic modification of the *agouti* gene expression (154). Finally, inherited disorders such as Rett syndrome (6) and "immunodeficiency, centromere instability, and facial dysmorphism" (ICF syndrome) (66, 116, 157) result from genetic errors in proteins or enzymes that play a role in methylation-regulated gene expression or genomic stability. Whether dietary manipulation in these or in imprinting disorders may improve the phenotype is under investigation (8). From evaluation of the metabolic pathways that lead to the synthesis of the methyl donor SAM, it can be derived that folic acid, betaine, zinc, and vitamin B12 have the potential to affect methylation, and any combination of these supplements could be construed to be a "methylation diet." Other factors in the metabolic pathway, such as methionine or SAM itself, could also be directly manipulated.

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THE BASICS: BIOLOGY OF GENOME METHYLATION

DNA Methylation Patterns

Over 80% of the cytosine residues in CpG dinucleotides of mammalian genomic DNA are methylated at position 5. Methylation is not equally spread throughout the genome. It is notably absent in CpG-rich regions near the 5′-end of genes, called CpG islands. These commonly contain the promoter regions of genes, and their unmethylated state correlates with active transcription (21, 23). When the promoter-associated CpG islands are methylated, transcription is irreversibly suppressed, as in the silenced allele of imprinted genes or genes on the inactive X chromosome. Most CpG-island methylation is stably inherited by daughter cells during mitotic cell division, but variations for a number of genes have been demonstrated in aging and cancer, and these may have significant biological consequences (42).

Methylation Enzymes

Three biologically active DNA-methyltransferases (DNMT1, DNMT3a, DNMT3b) are capable of adding methyl groups to the cytosine in CpG dinucleotides (18, 19, 74, 117). All DNMTs are essential for embryonic development. DNMT3a and 3b are de novo methylases, whereas DNMT1 is a maintenance methylase with preferential activity for hemi-methylated DNA (95, 96, 116, 117). DNMT1 may also have weak de novo methylation activity (74, 117), and specific variants of this protein (Dnmt1o) could participate in the establishment of methylation patterns and allele-specific expression of imprinted genes (73). The biological activity of two other proteins with DNMT similarity (DNMT2 and DNMT3L) has not been confirmed yet (3, 49, 118, 160).

DNA Methylation and Transcription

DNA methylation of CpG islands in promoter regions can, theoretically, interfere with transcription in three ways (88). It can interfere with binding of transcription factors, but some factors can still bind when the DNA is methylated. Methylated DNA can interact with protein complexes such as MeCP1 and MeCP2 that specifically bind to methylated CpG dinucleotides (29, 103, 104, 109, 112), or it could alter chromatin structure to a repressive state (7, 89). It has now become clear that these three mechanisms are closely interrelated (see below). Not all CpG islands associated with genes are in the 5′ regulatory regions but can be found in introns, exons, and 3′ regions (85, 93). Methylation of these CpG islands is not necessarily associated with transcriptional repression of the associated genes. If the CpG islands belong to suppressor elements that become inactivated by methylation, the result could in fact be increased and not decreased expression (83).

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The Methyl CpG-Binding Proteins

The mechanistic link between CpG-island methylation and transcriptional repression remained elusive until the isolation and functional characterization of a family of methyl-CpG-binding domain containing proteins (MBDs) (68). MBD2 is part of the methyl-CpG-binding complex 1 (MeCP1), which requires multiple consecutive methylated CpG dinucleotides for binding (104, 114). In contrast, the methyl-CpG-binding protein 2 (MeCP2) binds with high affinity to single symmetrically methylated CpG dinucleotides (110, 112). Seminal experiments by Jones et al. (86) and Nan et al. (111) demonstrated convincingly that binding of MeCP2 to methylated promoter constructs is associated with the recruitment of transcriptional repressor complexes containing histone deacetylase 1 and 2 (HDAC1, HDAC2) and corepressors such as Sin3A, establishing the molecular link between DNA methylation and gene silencing. Histone deacetylases belong to a family of proteins that are part of different transcriptional repressor complexes. Their enzyme activity leads to deacetylation of specific lysines in the tails of histones associated with nearby DNA, which compacts chromatin into a transcriptionally repressive "heterochromatic" state (48, 113).

The third MBD protein (MBD3) belongs to the core NuRD repressor complex but does not bind with significantly higher affinity to methylated DNA, and it is still unclear whether MBD3 targets this complex to methylated DNA (104, 164). It is possible that the factor that targets the NuRD complex to methylated DNA is MBD2, which also interacts with this complex (69, 164). Methylated cytosines are prevalent throughout the genome and are highly mutable residues that easily undergo deamination to form uracyl. This process needs an efficient repair mechanism to protect the genome from mutations. MBD4 is a DNA glycosylase that belongs to a complex that binds to these deaminated methyl-cytosines to repair them (70).

Is There Active DNA Demethylation?

Demethylation appears to be rare in differentiated cells and usually occurs via a slow, passive process when cells fail to remethylate previously methylated sequences after DNA replication (100, 131). This could occur when methyl donors are in short supply. Active and rapid demethylation is known to occur only in the zygote's paternal genome immediately after fertilization, preceding the demethylation of the maternal genome (107, 120). One report suggested that an alternatively spliced product of MBD2 is a demethylase (20), but these findings were never replicated in subsequent studies (114, 149), and an active demethylating enzyme has yet to be found (155).

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Histone Modifications

Methylation of DNA is associated with a repressive state of chromatin, brought upon by deacetylation of lysines in the tails of the core histones H3 and H4. Deacetylation of histone tails is a common mechanism for transcriptional repression (113). Histone acetylation is associated with transcriptionally active chromatin, and histone acetyltransferases are commonly found in transcriptional activator complexes. Additional modifications of histones, such as phosphorylation and methylation, play a role in transcriptional regulation as well. Interestingly, a connection between histone methylation and acetylation is one mechanism that can regulate transcription (10, 115). The histone methyl transferase enzymes also use SAM as the moiety that brings in a methyl group. Therefore, one can hypothesize that dietary factors that affect the supply of methyl groups can affect genome function at this level in addition to having direct influence on cytosine methylation.

What is the Biological Function of DNA Methylation?

Methylation is essential for survival in mammals: Null mutations in any of the three *Dnmts* in mice are lethal (96, 116). DNA methylation is important for the irreversible and mitotically heritable silencing of genes on the inactive X-chromosome and for the parent-of-origin dependent silencing of one allele of imprinted genes (23). However, because most DNA methylation occurs outside the regions that play a role in direct regulation of gene activity, it must have other functions. It has been proposed to be a repression mechanism used in complex organisms to reduce transcriptional noise (22). At least 35% of the genome consists of scattered endogenous transposon DNA, which can be regarded as highly specialized intragenomic parasitic DNA. In some cases (L1 repeats, for example), these can encode all necessary factors for transcription and need to be silenced, as their transposition can disrupt functional genes or induce chromosomal rearrangements (161). This silencing is achieved by high levels of methylation of these sequences.

There is as yet very little evidence that (reversible) promoter methylation is an important mechanism for the control of expression of developmentally regulated

genes (161), and some data argue against this. Ectopic expression of tissue-specific genes in mouse embryos that have no functional *Dnmt1* enzyme has not been observed. Walsh & Bestor studied several developmentally regulated genes in embryos and newborn mice and found no evidence that promoter methylation correlates with gene expression (151). It is true that embryonic globin is methylated during development, and this correlates with transcriptional repression (57, 140); but again, not all globin genes are methylated in nonexpressing tissues. The MyoD gene is important in muscle development and is turned off in other tissues, but its inactivation also fails to correlate well with methylation (85). Promoter methylation may be used only to strengthen the repression of developmental genes that would have deleterious effects if inadvertently reactivated.

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THE DNA SYNTHESIS AND METHYLATION PATHWAYS: ROLES OF FOLATE, BETAINE, VITAMIN B12, AND ZINC

The Metabolic Pathways

SAM is the major methyl donor for most cellular methylation processes. As shown in Figure 1, SAM is synthesized in the methylation pathway directly from the essential amino acid methionine. There are currently three enzymes known to be necessary for synthesis of methionine. The most important, methionine synthase (MTR), transfers a methyl group from 5-methyltetrahydrofolate (5MTHF) to homocysteine by reduction of 5MTHF to tetrahydrofolate (THF). This enzyme is ubiquitously expressed and active in all tissues and organs. Methionine synthase is one of two enzymes (the other is methyl-malonyl CoA-mutase) that require vitamin B12, as methylcobalamin (mB12), as a cofactor. Folic acid enters this pathway via dihydrofolate (DHF) and can be recycled through the formation of 5,10-methylene-tetrahydrofolate (5,10MTHF). Serine and glycine are the main donors of the carbon-1 groups in this process. When 5,10MTHF is reduced to 5MTHF, it releases methyl groups that are used in thymidilate and purine biosynthesis. Folic acid is therefore not only important for the methylation of genomic DNA, but also for its synthesis.

Fifty percent of methionine in the liver is synthesized via an alternate pathway, when betaine is hydrolyzed to dimethylglycine by the enzyme betaine-homocysteine methyl transferase (BHMT), which in most mammals is expressed only in liver and kidney (52, 101). Betaine itself is derived from the oxidation of choline. Recently, a second betaine-homocysteine methyltransferase (BHMT2) was identified (35), which is most abundant in adult liver and kidney but is also present at lower levels in other tissues (brain, heart, skeletal muscle) and during development (mouse fetal heart, liver, lung, kidney, and eye). Both BHMTs are zinc-dependent enzymes.

When SAM donates its methyl group to DNA, RNA, proteins, or phospholipids as a cofactor for a variety of methyltransferases (including the DNMTs), it is converted to S-adenosylhomocysteine (SAH). The enzyme S-adenosylhomocysteine

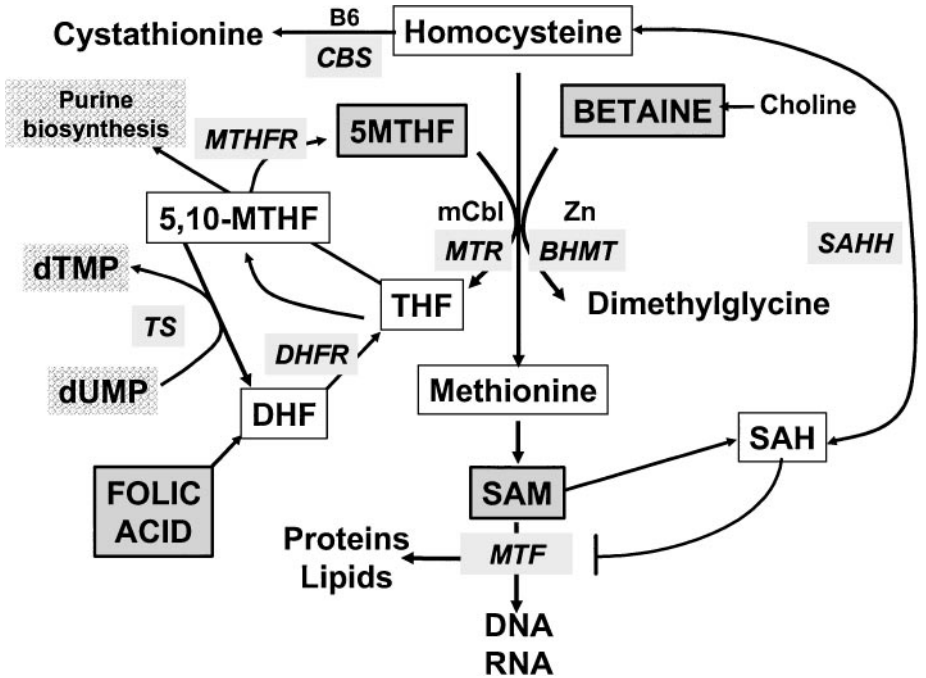

Figure 1 Methylation pathway. Homocysteine (top middle) is converted into methionine by two pathways: methionine synthase (*MTR*), which uses methylcobalamin (mCbl) as a cofactor and acquires a methyl group from the conversion of 5-methyltetrahydrofolate (5MTHF) into tetrahydrofolate (THF). 5MTHF is acquired via food folates or folic acid supplementation which is converted from dihydrofolate (DHF) by dihydrofolate reductase (*DHFR*) and 5,10-methylenetetrahydrofolate (5,10MTHF) by methylene tetrahydrofolate reductase (*MTHFR*). These also participate in DNA synthesis through conversion of dUMP in dTMP by thymidilate synthase (*TS*) and purine biosynthesis. In selected tissues, methionine can also acquire a methylgroup via conversion of betaine to dimethylglycine by betaine homocysteine methyltransferases (*BHMT*) which use Zinc (Zn) as a cofactor. Methionine is further converted to s-adenosylmethionine (SAM), the major methyl donor for all methyl transferases (*MTF*), which add methyl groups to DNA, RNA, lipids, and proteins. SAM is recycled via the intermediate s-adenosylhomocysteine (SAH) which is converted to homocysteine in a reversible reaction by s-adenosylhomocysteine hydrolase (*SAHH*).

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hydrolase (SAHH) converts SAH to homocysteine, leading to recycling of homocysteine and methionine in the remethylation pathway. Inadequate supply of methyl groups owing to lack of methyl donor agents such as folate or betaine, or deficiency of the cofactors methylcobalamin and zinc, can result in accumulation of homocysteine and reduced levels of SAM. Homocysteine is also converted to cysteines in the transsulfuration pathway. SAH hydrolysis is a reversible reaction,

and when homocysteine accumulates, the result can be increased levels of SAH and decreased SAM/SAH ratios (the methylation index). SAH has a direct inhibitory effect on the DNA-methyltransferases. Defects in enzymes participating in these connected pathways or inadequate dietary supply of cofactors and substrates, with accumulation of precursors, can be associated with changes in DNA methylation. Most studies have focused on clinical and subclinical deficiencies that decrease DNA methylation. The potential therapeutic benefits of providing increased supplies of these intermediates have only recently begun to be investigated.

Exogenous Agents that can Affect DNA Methylation

Some cancer research has shown, both in vitro and in vivo, that the methylation of specific CpG islands can be changed in differentiated cells, with hypermethylation repressing tumor suppressor genes or hypomethylation activating proto-oncogenes (82, 166) (see below). These events have been successfully influenced in cell culture assays and in laboratory animals by methyl donor supplementation and depletion (41, 121, 150, 166). Because the universal methyl donor used by DNA methyltransferases to add 5-methyl groups to cytosine is S-adenosylmethionine (SAM), varying the levels of any of the factors that feed into this methylation pathway could increase or decrease DNA methylation efficiency.

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FOLIC ACID AND ITS DERIVATIVES Reduced folates act as cofactors for many processes by carrying one-carbon units that are required for numerous cellular methylation reactions and for the synthesis of glycine, thymidilate, purines, and methionine. Food sources contain a mixture of reduced folates attached to a polyglutamate (dihydrofolate or tetrahydrofolate). These compounds are very unstable and have only 25–50% bioavailability. During absorption by the intestinal mucosa, the polyglutamate chain is removed and, if not already present, the 5-methyl group is added to form 5MTHF. Folate supplements contain the synthetic form, folic acid, which is stable for months to years and has a bioavailability of 100%. During its absorption in the intestine, folic acid is reduced by the enzyme dihydrofolate reductase to dihydrofolate and tetrahydrofolate and then converted into 5MTHF. Once inside the cell, it becomes polyglutamated again to assure that it is retained. Dihydrofolate reductase has limited capacity, and when excess folic acid is ingested from supplements, it can appear unaltered in plasma. Some can then be taken up and metabolized by other cells if they have active dihydrofolate reductase (for review see 135). The enzyme methionine synthase requires 5MTHF. Under normal circumstances, the DNA synthesis and methylation cycles regenerate THF, but a small amount is lost in urine, bile or via the skin, and if not replenished a functional deficit can occur (135). The cell therefore needs to take up circulating serum folate (MTHF monoglutamate) (132) (see Figure 1). The levels of SAM and SAH regulate THF formation, so that when SAM is low or SAH is high, methylene tetrahydrofolate reductase (MTHFR) is activated and folate directed from DNA synthesis to the remethylation pathway (158). Folate is a water-soluble vitamin

and generally considered safe, but laboratory rats supplemented with very high doses had smaller offspring (4), and doses of 200 mg/kg can cause acute renal failure in mice (33).

BETAINE AND CHOLINE The second methyl donor, betaine, enters the pathway through choline oxidation. All betaine in the body is derived from choline and, unless supplemented, none is taken up from the diet. Betaine supplementation has proven very valuable for disorders caused by defects in the folate recycling pathway (e.g., MTHFR deficiency). Betaine is hydrolyzed by BHMT1 or 2 to dimethylglycine when it transfers its methyl group to homocysteine to generate methionine (35, 52, 101). BHMT1 and 2 have limited organ distribution, so it remains unclear whether the neurological benefits of betaine therapies result directly from homocysteine conversion in the brain or indirectly from improved conversion in other organs (101). Choline is an essential dietary component and an important metabolite for several other aspects of cellular and neuronal function. It is a major component of phospholipids and sphingomyelin and participates directly in neurotransmission as acetylcholine (162). Choline-deficient diets in rats lead to a decrease in SAM in their tissues (163).

VITAMIN B12 (METHYLCOBALAMIN) Methionine synthase (MTR) is one of two cobalamin-dependent enzymes in humans. MTR uses methylcobalamin, whereas the other vitamin B12–dependent enzyme, methylmalonylCoA mutase, uses adenosylcobalamin. Vitamin B12 absorption from food requires the cooperation of the acidic milieu of the stomach, binding proteins, and intrinsic factor. B12 also is recycled via the enterohepatic circulation. Several acquired and inherited conditions can lead to vitamin B12 malabsorption and errors in cobalamin metabolism (135). Severe cobalamin deficiency leads to pernicious anemia, neuropathy, and demyelination. Because of the B12 requirement of MTR, all folate supplementation should be accompanied by adequate methylcobalamin levels if the goal is to improve DNA methylation reactions (154).

Zinc is essential for many enzymes that affect DNA stability, synthesis, repair, and gene transcription in the form of zinc finger proteins, but under normal circumstances deficiencies are rare (50). BHMT is also a zinc metalloprotein (35, 105). Diets designed to improve methylation may therefore require zinc supplementation (154).

METHIONINE Methionine is an essential amino acid that is not only needed for methylation reactions as the immediate precursor of SAM, but is also required for protein synthesis. Because most methionine is recycled from homocysteine via the remethylation cycle, the food supply usually provides an excess of 60% (135). Thus, when the remethylation reaction is inadequate, the functional levels of methionine can become too low, resulting in lower levels of SAM. Dietary supplementation with methionine reduces neural tube defects in the axial defects (*Axd*) mouse model (51), while methionine requirements are high during pregnancy

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to supply the increased protein synthesis associated with rapid fetal growth. Its suboptimal status could play a role in folate-responsive neural tube defects (124).

S-ADENOSYLMETHIONINE SAM is formed from adenosine and methionine by methionine-adenosyl transferase. Tight regulation of a continued adequate supply of SAM may be the major goal of the remethylation pathway (26). Oral and parenteral administration of SAM can restore its blood and cerebrospinal fluid levels, and it appears to move across the blood-brain barrier (127). Its use has been investigated in depression (26), liver disease (119), and osteoarthritis (30), as well as in inherited disorders of folate metabolism (143). Direct administration of SAM could be investigated as a therapeutic strategy to improve DNA methylation processes.

HOMOCYSTEINE AND S-ADENOSYLHOMOCYSTEINE All homocysteine is derived in vivo from recycling of SAH by the enzyme SAH hydrolase (SAHH). This is an important step in the regulation of the methylation reactions. It assures adequate supply of SAM via the remethylation cycle, but also efficiently removes SAH, which has higher affinity for the methyl transferases than SAM and can therefore act as a competitive inhibitor. When homocysteine levels become elevated because of defects in the transsulfuration pathway or remethylation pathway, the SAHH reaction, which is reversible, results in accumulation of SAH, and a lower SAM/SAH ratio or methylation index. Yi et al. have shown that this can occur in mildly elevated homocysteine states and propose that this is an important contributor to homocysteine toxicity (158).

The Methyl Folate Trap Hypothesis

Low vitamin B12 or insufficient methionine synthase limits the methionine supply necessary to generate the methylation donor SAM. This will stimulate MTHFR to generate more 5MTHF in the organism's attempt to recycle folate towards methionine synthesis. However, with inadequate methylcobalamin function, the 5MTHF accumulates, while the intracellular folate pool available for methylation reactions decreases. The effect of vitamin B12 deficiency on this pathway is thus a functionally deficient folate state with hyperhomocysteinemia and a low methionine level (136).

Interaction with Genetic Defects

Defects in several of the enzymes in the folate and remethylation pathway can result in severe inherited metabolic disorders (described below). Common mutations or polymorphisms leading to mildly decreased enzymatic function that have been clearly associated with an increased risk for common multifactorial disorders (primarily cardiovascular disease and cancer) and birth defects (primarily neural tube defects) are summarized here. Detailed discussions of the incidence of these variations and their association with common disorders can be found elsewhere (9, 28, 90, 134).

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MTHFR POLYMORPHISMS: The gene for MTHFR was isolated in 1994 (61). In 1995 a common thermolabile polymorphism (677C>T, which changes alanine to valine) that reduced its activity was found to be associated with hyperhomocysteinemia (61), especially in individuals whose diets are low in folate (133). This polymorphism has been investigated as a risk factor for cardiovascular disease (31), birth defects (28), and cancer (36, 37, 99). It appears that the risk elevation is most significant when the 677C>T polymorphism is associated with low folate intake (79). A second polymorphism (1298A>C, which changes glutamate to valine) does not alter homocysteine levels by itself, but in the compound heterozygote state with 677C>T can behave similarly to the homozygous 677C>T mutation (28).

METHIONINE SYNTHASE POLYMORPHISMS A common polymorphism in MTR is 2756A>G, which results in an aspartate to glycine change at amino acid 919 (38) and has been investigated as a risk factor for cancer, cardiovascular disease, and spina bifida. Its contribution may also be influenced by low folate (153).

METHIONINE SYNTHASE REDUCTASE POLYMORPHISMS Methionine synthase reductase is necessary to maintain MTR in an active state (94). A common variant, 66A>G (isoleucine to methionine), has been associated with an increased risk for spina bifida in one study (153), and may contribute to increased risk for offspring with Down syndrome (72).

DIETARY INFLUENCES ON DNA METHYLATION AND HUMAN DISEASE

Cancer

In comparison to normal differentiated cells, tumor cells are characterized by a global hypomethylated state despite overexpression of DNA methyltransferase, although they have distinct regions of hypermethylation (16, 84). Even though this process and the influence of inadequate dietary supply of methyl donors and/or genetic predisposition to aberrant methylation have been extensively studied, the precise mechanisms underlying the methylation aberrations in cancer are poorly understood. Neither is it known whether global hypomethylation and regional hypermethylation are causally linked or are completely independent events occurring in the same cellular context of malignant transformation (42). The prevailing hypothesis is that hypermethylation of promoter-associated CpG islands of tumor suppressor genes leads to inactivation of these genes and development or progression of cancer (16). A large number of studies have been performed on various primary tumors and tumor cell lines. Promoter hypermethylation has been observed in genes that play a role in cell cycle regulation, apoptosis, DNA repair and replication, angiogenesis, cell differentiation, and other processes that, when

uncontrolled, can promote carcinogenesis (for review see 42). Silencing these genes can be a second hit in the two-step carcinogenesis process (62). This was first demonstrated for the calcitonin gene and *MYOD1* (85), and later followed by *RB1* and numerous other well-known tumor suppressor genes, such as *VHL*, *p16*, *E-cadherin*, and *hMLH1*. Interestingly, these methylation changes do not always correlate with a role for the specific gene in the given cancer, suggesting that they are random events that can then be clonally selected because of a growth advantage of the cell. Hypermethylation is potentially reversible, and treatments with methylation inhibitors are being investigated in particular neoplasias, such as leukemia (15).

Global DNA hypomethylation has been measured in many cancer cells and primary tumors from humans and animal models. There are several hypotheses to explain the carcinogenic effects of DNA hypomethylation (42). Activation of latent endogenous retrotransposons could disrupt genes close by (or distant, after retrotransposition to other areas). Chromosomal instability and DNA double-strand breaks are other possible mechanisms that have been demonstrated in laboratory rats fed hypomethylated diets (91), in postmenopausal women with methyl-donor deficient diets (78), and in cancer cells. Lastly, hypomethylation could lead to activation of oncogenes. In vivo studies of rats fed methyl-donor deficient diets showed that overexpression of genes such as *c-myc*, *c-fos*, and *c-ha-ras* and in liver-derived RNA correlated with hypomethylation of DNA and the duration of the diet (41, 150). Interestingly, when diets were methyl-donor replete, the DNA methylation reverted to normal, but not for all cytosines that were examined. This suggests that DNA methylation defects can be irreversible after prolonged exposure to diets low in methyl donors (41).

Increased sensitivity of rats to known carcinogens has also been observed when diets are methyl-donor deficient (129). In humans a good correlation between reduced tissue and plasma folate levels and hypomethylation of both normal and tumor tissues was demonstrated in patients with cervical cancer (55). A large prospective study in humans also showed that methyl donor deficiency correlates with an increased risk for tumors of the liver and colon (58, 59) and demonstrated an inverse relationship between DNA methylation of colon adenomas and dietary folate. Such studies further suggest that this risk may be aggravated by other risk factors, such as alcohol intake and smoking. Polymorphisms in the folate metabolic enzymes, e.g., the MTHFR 677C>T thermolabile mutation, also influence cancer risk (99). Interestingly, adequate folate intake significantly decreased the risk for these colon tumors in homozygous 677T/T individuals; this may be explained by a protective effect on the DNA synthesis branch of the folate pathway.

Because of the variability in methylation within one tumor or between tumor types, the potential benefit of methylation diets in cancer treatment or prevention, versus treatment with demethylating agents, may be highly variable. Further investigations focusing on cancer staging and determination of the methylation profiles of individual tumors will be needed to answer this question.

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Aging

There is a correlation between age and global DNA hypomethylation (71, 125). If aberrant DNA methylation and cancer are causally linked, it may contribute to the age-related increase in cancer risk. The first study of gene-specific promoter hypermethylation investigated methylation in the promoter region of the estrogen receptor in colon mucosa. This study revealed a linear increase with age in promoter methylation of this gene of about 1% over 4 years (77). Methylation profiling of cancerous and noncancerous tissues at various ages, performed by the same investigators, revealed similar patterns for a significant number of genes and a correlation between age-specific methylation and cancer development (145). However, they found that some genes never undergo age-related methylation but only aberrant methylation in cancer (145). As with the situation in cancer, it is not understood whether there is a causal relationship between global hypomethylation of DNA and hypermethylation of specific sequences during aging. Therefore, dietary manipulations to increase levels of methyl donors should be evaluated within an individual's genomic context.

Cardiovascular Disease

Although this topic remains controversial, there is an association of elevated homocysteine levels with increased risk for atherosclerosis and venous thrombosis, supported by epidemiologic evidence. A recently generated mouse model with suboptimal function of MTHFR shows increased venous and arterial thrombosis (40). It has been proposed that the development of atherosclerosis is caused by homocysteine toxicity, but DNA methylation defects may be at least partially responsible (158). Age-related methylation changes could also contribute to this process. Post et al. compared the methylation status of the estrogen receptor in atherosclerotic plaques with normal vascular tissue from patients undergoing coronary bypass grafts or directed coronary endarterectomy (123). They found that estrogen receptor hypermethylation was significantly more prominent in the atherosclerotic plaques and increased in cultured aortic smooth muscle cells (159). This could play a role in development of coronary artery disease and other forms of atherosclerosis and provide an explanation for the lack of benefit of estrogen replacement therapy after the onset of atherosclerotic disease (123).

Mental Function

DEPRESSION AND OTHER PSYCHIATRIC DISORDERS Several lines of evidence exist for a high dependence on methylated cytosine for normal development and function of the central nervous system. Expression of DNA-methyltransferases (DNMTs) and methyl-CpG-binding protein 2 (MeCP2) is high in developing brain but decreases later (44, 128, 137). Mutations in *MECP2* cause the neurodevelopmental disorder Rett syndrome as well as some cases of mental retardation and autism (6, 43, 92, 138). Mice lacking MBD2 have a neurobehavioral

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phenotype, and mice lacking *Dnmt1* only in the brain lose neurons and die after birth (69).

A subgroup of patients with folate deficiency have neuropsychiatric symptoms such as fatigue, depression, and altered intellectual capacity. Studies in laboratory animals and patients correlated folate-responsive depression with low levels of serotonin or its metabolites 5-hydroxyindoleacetic acid and homovanillic acid (24). Similar findings were seen with vitamin B12 deficiency (25). It was further hypothesized that the similarities between folate and B12-responsive neuropsychiatric symptoms might be related to changes in methylation processes within the central nervous system (127). This was substantiated by low levels of S-adenosylmethionine (SAM) in folate-responsive depression (27). Oral or parenteral supplementation with SAM has been found to be a valuable antidepressant in endogenous depression, in Parkinson disease-associated depression, and in depression associated with anticonvulsant therapy in small trials (26). All these observations suggest that methylation reactions in the brain are altered with low levels of SAM, which can be influenced by methyl donor diets.

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MEMORY A study of rats demonstrated that supplemental choline given to pregnant rats improved long-term memory of their offspring, suggesting that such therapy might have a long-term benefit for neuronal function (102). It was further demonstrated that it is not the neurotransmitter acetylcholine, but phosphocholine and the choline oxidation product, betaine, that accumulate. This suggests that an increase of this methyl donor is important in this process, whereas other intermediates of the choline metabolic pathway, such as acetylcholine and sphingomyelin, appear to be unchanged (56, 162).

Inherited Disorders of Folate and B12 Metabolism

Patients with inborn errors of folate metabolism are very rare but can provide insight into the role of methylation processes in health and disease. Clinical MTHFR deficiency causes homocystinuria, characterized by subacute combined spinal cord degeneration and central nervous system symptoms with demyelination. This disorder is associated with low folate, methionine, and SAM levels; symptoms can be reversed by administration of betaine as an alternative source of methyl groups for the remethylation of homocysteine to methionine (75). A rare patient with methionine adenosyl transferase deficiency had high levels of methionine and low SAM, which was reversed with SAM treatment (143). All these therapies led to partial reversal of demyelination.

Because DNA methylation in the brain has not been formally investigated in any of these central nervous system conditions, its contribution to the symptomatology, compared to that of deficient methylation reactions during synthesis of proteins, phospholipids (myelination), and neurotransmitters, remains to be seen. Mice with homozygous MTHFR deficiency have been generated and have central nervous system and vascular abnormalities, hyperhomocysteinemia, global

DNA hypomethylation and vascular lipid deposition (40). Absence of methionine synthase in mice is embryonic-lethal, while heterozygotes have mildly elevated homocysteine, but methylation studies were not reported (144). Lastly, mice deficient for the liver-specific methionine adenosyl transferase 1A have lower hepatic SAM and glutathione, but interestingly, the levels of S-adenosylhomocysteine (SAH) and DNA methylation were unchanged (97).

Neural Tube Defects and Other Birth Defects

Folic acid prevents 70% of neural tube defects (NTD); periconceptional folic acid supplementation (>400 μg/day) clearly reduces the occurrence and recurrence of NTD (1, 17, 46). The mechanisms by which folate supplementation prevents NTD are largely unknown, but both the effects of folate on DNA synthesis and methylation may play a role. Recent studies of spontaneous mouse mutants with NTD and single-gene knockout mice with NTD have started to elucidate the involved pathways and genomic changes [for an excellent review see (87)].

Fleming & Copp found that increased incorporation of [^{3}H] thymidine and frequency of NTDs in cultured *Splotch* (homozygous *Pax3* mutation) embryos responded to folic acid and thymidine but was exacerbated by methionine (53), suggesting an abnormality in folate metabolism. This strengthens the argument that folic acid is unlikely to prevent NTDs by causing increased abortion of affected fetuses, as previously proposed, but the molecular effects of folic acid supplementation and changes in DNA methylation were not investigated in this work. Other mouse mutants with NTDs, such as heat-induced NTDs, the null mutation of the *Cart1* homeobox gene, *crooked tail*, and a null mutation of the folate binding protein 1 (*Folbp1*) respond to folic or folinic acid (34, 122, 139, 165). It is probable that the defects in any of these may result from dysregulation of DNA methylation and/or DNA synthesis, but formal studies addressing this question have not been reported. Although experiments in avian embryos suggested an elevation of homocysteine as the primary toxic factor causing NTDs and cardiac defects (130), subsequent analysis on mouse embryos could not substantiate this observation (65).

A number of genes that are either orthologues of those associated with NTDs in mice (142) or play a role in folate metabolism (11, 12) have been investigated in human NTD patients, but no statistically significant associations were uncovered. Most studies have focused on MTHFR polymorphisms and have yielded variable results, ranging from no association to a seven times greater risk for the 677C>T thermolabile polymorphism. A recent meta-analysis quoted an overall twofold risk (28).

Conotruncal defects and facial clefting may also be influenced by folic acid (47, 106). Their sensitivity to folate levels may be explained by their embryogical derivation from neural crest epithelium, which also gives rise to the neural tube (130).

The exact mechanisms underlying the benefits of folic acid supplementation in the prevention of NTD are still unknown. Investigations have focused on

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deficiencies in critical enzymes in the folate-homocysteine-methionine pathway, the role of folate in various aspects of C1-metabolism, the role of folate as a methyl group–donor for purine and thymine biosynthesis, and deficiencies in the folate transport mechanism (53, 122, 132, 147, 148). A role for altered DNA methylation in NTDs has been suggested but not formally investigated (80, 132).

Human Aneuploidy and DNA Methylation

Because there is evidence that chromosomal instability and aneuploidy in tumors may be related to genome-wide DNA hypomethylation, James et al. argued that DNA hypomethylation could lead to meiosis I nondisjunction and subsequent aneuploidy, such as trisomy 21 (80). They evaluated the allele frequency of the common thermolabile MTHFR polymorphism (677C>T) in women who had given birth to children with Down syndrome and correlated it with their plasma homocysteine, methionine, and genomic DNA methylation levels. They found that these women had higher frequencies of the 677T allele, higher plasma homocysteine levels, and reduced DNA methylation. This work was expanded to a larger group of women, also taking into account the allele frequency of the methionine synthase reductase 66A>G polymorphism. The previous results were confirmed, and the new data further suggested that both polymorphisms synergistically increase the risk (72). However, an independent study evaluating other aneuploidies that could have resulted from meiosis I nondisjunction did not confirm the association with the MTHFR and methionine synthase reductase polymorphisms (67).

There is also indirect evidence that the periconceptional folic acid supplementation implemented to reduce the risk for NTDs has been associated with a lower incidence of Down syndrome (2, 46). Because elevated homocysteine levels in the studies by James et al. (80) and Hobbs et al. (72) did not track with the polymorphism genotypes, they argued that these patients might have a genetically determined increased requirement for folate that makes them less tolerant to suboptimal dietary levels. One weakness of all these studies is that the biochemical assays were not performed around the time of conception, and there is certainly a need for large prospective trials to confirm or disprove these results.

Regulation of Developmental Gene Expression and Long-Term Effects

The proper activation of developmental genes during embryogenesis and later fetal development is a highly regulated process that operates under the constant influence of environmental and internal factors such as retinoids, oral anticoagulants, or antiepileptics, or through the accumulation of endogenous substances such as elevated levels of glucose (and insulin) in diabetes mellitus (152). These factors can increase the risk for specific birth defects through known interactions with defined metabolic pathways, such as in the case of retinoids (152), but the specific biological mechanisms involved are for the most part not well understood (1, 17, 32, 47, 106).

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A significant number of epidemiological studies have also shown that nutritional balance during intrauterine life influences not only fetal and childhood development but also the risk for common adult-onset disorders (13, 14, 60). Global nutritional deficiencies during pregnancy can increase risk to the offspring for hypertension, cardiovascular disease, obesity, and type 2 diabetes (152). Evidence for this has been obtained both from epidemiological investigations (13, 14, 45, 54, 126) and from animal research (81, 156). These observations imply that the cells have a "biological memory" for these environmental influences that can be passed on to daughter cells through mitotic and possibly meiotic cell division. Investigators have used the term "metabolic programming" or "metabolic imprinting" to describe this process, but the underlying biological mechanisms that regulate it are largely unknown (98, 152).

There are several theories to explain the molecular basis for this metabolic imprinting. Permanent changes in cell number, variations in organ growth and structure, clonal selection of specific cell types over others, or "metabolic differentiation," i.e., the development of a stable quantitative and qualitative pattern of basal and inducible gene expression, have all been proposed as biological mechanisms (152). This implies that, even though the genome of each cell of the body is the same, a mitotically or meiotically heritable mark or "epigenetic mark" on gene function is induced in a subset of cells under the influence of exogenous factors. DNA methylation is a good candidate for such an epigenetic mark, because it is both quasi-irreversible in differentiated cells and heritable in mitosis and meiosis. The role of changes in DNA methylation in the developmental regulation of gene expression is not well established at this time. One study, looking at methylation of the promoters of developmental genes during various embryonic stages, found no changes, and the authors concluded that methylation does not play a role in this process (151). On the other hand, mice with null mutations in DNA methyltransferases arrest in development (96, 116).

The first experiment to demonstrate that DNA methylation and gene expression in the offspring can be altered by maternal diet was performed by Wolff and colleagues on the *agouti* viable yellow (A^{vy}/a) mutant mouse line. These mice express the *agouti* gene under the control of a promoter in a viral intracisternal A particle, which, when methylated, suppresses the expression of the gene and causes a yellow coat, but in the unmethylated state leads to the expression of this gene and an agouti coat color. When these investigators compared pregnant mice on control diets with those on diets supplemented with folate, betaine, choline, methionine, zinc, and vitamin B12, they could change the expression and methylation of the intracisternal A particle associated with this gene and alter the coat color of the offspring (154). This change correlated with methylation of the intracisternal A particle sequence (CA Cooney, personal communication). The epigenetic inheritance seen in these animals (108, 154) suggests that these changes may be inherited by subsequent generations. The effect on memory of newborn rats of dams fed supplemental choline could be due to such a methylation-dependent gene expression alteration (56, 102, 162).

SPECIFIC DISORDERS OF DNA METHYLATION

ICF Syndrome

This rare autosomal recessive disorder is characterized by immunodeficiency, centromeric instability, and facial dysmorphism. Patients have recurrent infections of the skin and respiratory and digestive tracts and variable degrees of mental retardation. Phytohemaglutinin-stimulated lymphocytes from these patients have typical decondensation of juxtacentromeric heterochromatin of chromosomes 1, 9, and 16 and abnormal chromosome segregation leading to the formation of multibranched structures. There is strong hypomethylation of nonsatellite repeat DNA in these patients. In 1999, mutations in the gene encoding DNA methyltransferase 3B (*DNMT3B*) were identified (116, 157). This is the only human disorder known to be caused by germline mutations in a DNA methyltransferase. The different *DNMT* enzymes have distinct but partially overlapping functions, and large supplies of folic acid might influence the phenotype. To date, no studies are known that have addressed this possibility, and it might be difficult to obtain a conclusive answer, because only about 40 patients have been described in the literature.

Rett Syndrome

Rett syndrome is an X-linked dominant disorder, affecting mostly females who are apparently normal for the first 6–18 months of life, after which they stop achieving intellectual milestones, suffer deceleration of head growth, loss of speech and purposeful hand use, and develop stereotypic hand movements, gait apraxia, seizures, autonomic dysfunction, and breathing abnormalities (64). The gene mutated in Rett syndrome encodes methyl-CpG-binding protein 2 (MeCP2) (6). This protein is the first identified mechanistic link between DNA methylation and transcriptional repression (86, 111), and we hypothesized that the pathogenesis of Rett syndrome may be related to inadequate transcriptional suppression of certain genes at critical stages during development of the central nervous system (6, 146). Ongoing studies utilizing animal models are addressing this hypothesis (39, 63; M.D. Shahbazian & H.Y. Zoghbi, unpublished data). We are now investigating whether Rett syndrome patients might benefit from dietary manipulations that increase DNA methylation in the hope of improving the function of a partially inactive MeCP2 protein or attracting any of the other methyl-CpG-binding domain (MBD) containing proteins to the hypermethylated CpG islands in promoter regions.

Imprinting Disorders

Imprinting results from the unequal expression of the maternal or paternal allele of a small number of genes in the genome (about 50 imprinted genes have been identified). This allele-specific transcriptional repression is achieved for most genes by differential methylation of promoter-associated CpG islands or nearby imprinting control regions. This methylation mark is established during gamete development

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and is stably transmitted during subsequent mitotic cell divisions. Most genes in the body are imprinted in all tissues, but some have tissue-specific imprinting, with relaxation of imprinting and bi-allelic expression in only a minority of tissues (such as *IGF2* in the choroid plexus and adult liver and *KVLQT1* in heart) or, as in Angelman syndrome, with bi-allelic expression in all tissues except the brain (which expresses only the maternal allele) (5). These observations suggest that for such loci, DNA methylation patterns might not be stable and irreversible, and could be responsive to dietary manipulation with methyl donor supplements (8).

AREAS FOR FUTURE RESEARCH AND CONCLUSIONS

The association between suboptimal methyl-donor supply, DNA methylation and disease is well demonstrated. More work will be needed to ascertain the relative importance of each methyl-donor in the metabolic pathway. Basic molecular mechanisms linking the changes in DNA methylation to the pathogenesis of disorders such as cancer and cardiovascular disease are still poorly understood, but DNA methylation is an attractive modification that can be used for rapid and permanent adaptation to a changing environment. An emerging area of research addresses the role of DNA methylation in metabolic programming or the prenatal environmental influences on (developmental) gene expression that have long-lasting effects. The role of methylation diets to alleviate symptoms of some genetic disorders of DNA methylation and prevention of birth defects is another important emerging field.

Are Current Dietary Recommendations for the Described Agents Adequate?

Recommendations for adequate dietary supply of vitamins and micronutrients were mostly designed to provide levels that are sufficient to prevent overt clinical symptoms of deficiencies.

The examples presented here illustrate that even small decreases in dietary factors that can influence methylation could have significant long-term health effects that accumulate over many years. This may contribute to common disorders of adulthood, such as cardiovascular disease and cancer, and possibly also affect early development. There is thus a need for large studies to reevaluate dietary recommendations for such nutrients. Most of the population in industrialized countries benefits from public health policies of dietary folate supplementation in fortified foods, but access to these nutrients can be limited by dietary customs, not to mention inadequate food supply, in many areas of the world. Furthermore, recommended supplementation for methyl-donor compounds may need to be adapted to individual genotypes and exposure to other risk factors. For example, individuals with polymorphisms leading to reduced activity of enzymes in the folate and methylation pathway may need higher supplements, as may individuals with higher exposure to certain carcinogens. Knowledge about functional polymorphisms in genes in these pathways or in modifier genes, as well as about individual DNA

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methylation profiles, will continue to accumulate in this era of genomic medicine. Such developments may improve longevity and decrease developmental and age-related disease.

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