Diet, DNA Methylation Processes and Health

Executive Summary

Introduction

The trans-HHS workshop on "Diet, DNA Methylation Processes and Health" was sponsored by the National Institutes of Health (NIH), the Food and Drug Administration (FDA), the American Society for Nutritional Sciences, and the International Life Sciences Institute, North America. This workshop provided an opportunity for basic scientists, clinical investigators, epidemiologists, and researchers from other disciplines to gain insight into current knowledge on the workshop topic and to encourage collaborations for future research.

Objectives for the workshop included:

- To increase awareness and understanding of the causes and mechanisms of abnormal methylation processes, including methyl metabolism and DNA methylation, in various physiological conditions involving growth and development, as well as in disease prevention; and
- To enhance knowledge and understanding about the role of dietary factors and genetic polymorphisms in methyl metabolism and DNA methylation processes.

Changes in DNA methylation are epigenetic changes -- that is, alterations of DNA that do not change the DNA coding sequence; evidence suggests that these changes can be inherited. In all species, epigenetic modifications of DNA appear to be important for genome function throughout life. DNA methylation processes have broad implications for human health and, with the advent of genomic studies that identify the effects of genes on disease states, it is likely that knowledge about methylation processes and changes in methyl metabolism and DNA methylation will prove to be significant in disease prevention and treatment. This Executive Summary provides the salient points covered by the speakers in each session of the Workshop and concludes with a compilation of the recommendations for future research that were presented during the final panel and general discussions.

Overview Topics

The precise control of genes, which constitute only a small proportion of the total mammalian genome, in the presence of an overwhelming background of non-coding bulk DNA, represents a problem in regulation. The presence of bulk DNA containing introns, repetitive elements and potentially active transposable elements, requires effective mechanisms for their long-term silencing. It appears that methylation of cytosine by S-adenosylmethionine (SAM)-dependent DNA methyltransferases to form 5-methylcytosine provides one such (heritable) mechanism for altering DNA-protein interactions. Genes can be transcribed from methylation-free promoters even though adjacent transcribed and non-transcribed regions are extensively methylated. This allows the promoters of organismal genes to be used and regulated, while keeping bulk DNA, including transposable elements, suppressed. Methylation is also used for long-term silencing of X-linked and imprinted genes and can either increase or decrease the level of transcription depending on where it occurs in the transcription unit. The potential role of methylation in tissue-specific gene expression, or in the regulation of CpG-poor

promoters, is less well established. Overview topics addressed in this Workshop session included DNA methylation in mammals, metabolic aspects of methyl group formation from one-carbon units, interactions among dietary folate, methionine, and choline, and the impact of nutrition, genetics, and chemical toxicity on aberrant DNA methylation.

Almost all normal DNA methylation in mammals is restricted to cytosine followed by guanosine (CpG) dinucleotide palindrome sequences which are controlled enzymatically by the DNA methyltransferases (Dnmt). Two different types of DNA methyltransferases have been characterized: de novo methyltransferases, e.g. Dnmt3a and Dnmt3b, that preferentially use non-methylated DNA as a substrate; and maintenance methyltransferases, such as Dnmt1, that preferentially methylate hemimethylated DNA that is generated by replication of methylated sites. Maintenance methylation implies the copying of the existing methylation pattern of the old DNA strand onto the new one. DNA methylation patterns can thus be heritable and serve as epigenetic marks that are transmitted by mitotic or meiotic cell division onto the progeny. Compared with other dinucleotides, CpG dinucleotides are under represented in vertebrate DNAs. They make up 1-2% of total dinucleotides, and they appear to be clustered in so-called CpG islands that are usually hypomethylated and often linked to promoter regions of genes. It is only after aberrant CpG island methylation that alterations in gene expression associated with cancer occurs. The consequences of such aberrant DNA methylation include silencing of tumor suppressor gene expression and cytosine (C) to thymine (T) transition mutations resulting from deamination of 5-methycytosine (5mC) to T. There is also evidence that appropriate chromosome structure may be affected by methylation and that human diseases including cancer are impacted by changes in chromatin structure.

Understanding the metabolic pathways of methylation related to nutrition is instructive for developing strategies to prevent methylation-related disease, including carcinogenesis. Recent evidence confirms that serine is the major source of one-carbon units for homocysteine remethylation and these one-carbon units can be generated in the cytosol and in the mitochondrion. Metabolic pathways of interest have been identified for remethylation, transmethylation, and transsulfuration. For example, methionine has a role in transmethylation pathways through enzymatic conversion to S-adenosylmethionine (SAM), which is demethylated to S-adenosylhomocysteine (SAH), further converted to homocysteine, and finally remethylated to methionine. Dietary deficiencies of the SAM precursors folate, methionine, vitamin B12, and choline have been associated with increased cancer risk. Of particular interest in the folate-methyl metabolic pathways is the enzyme methylenetetrahydrofolate reductase (*MTHFR*). Results from population studies indicate that in some populations polymorphic forms of *MTHFR* that result in lower levels of methylation have been associated with increased cancer risk. *MTHFR* knock-out mouse models have been developed to investigate this association. More research is needed to elucidate the relationship between *MTHFR* polymorphisms and cancer risk.

The metabolic transmethylation pathways closely interconnect choline, methionine, folate, as well as vitamins B6 and B12. These metabolic pathways intersect at the formation of methionine from homocysteine. Perturbing the metabolism of one of these pathways results in compensatory changes in the others and may result in one or more deficiencies of the transmethylation pathway specific nutrients. For example, methionine can be formed from homocysteine using methyl groups from N5-methyltetrahydrofolate (mTHF), or using methyl groups from betaine that are derived from choline. Similarly, mTHF can be formed from one-carbon units derived from serine or from the methyl

groups of choline via dimethylglycine, and choline can be synthesized *de novo* using methyl groups derived from methionine (via S-adenosylmethionine). The availability of transgenic and knockout mice has made possible additional studies that demonstrate the interrelationship of these methyl sources. *MTHFR* knockouts, which have impaired availability of methyl groups from mTHF, utilize (and consequently deplete) choline and betaine so as to maintain homocysteine remethylation. As we consider dietary requirements and possible effects on DNA methylation, it is important to realize that methionine, mTHF, and choline can be fungible sources of methyl-groups and the design of future studies should reflect this.

The impact of alterations in methylation related to methionine, mTHF, and choline deficiency is profound and has been associated with the major diseases of contemporary society, including cancer, cardiovascular diseases, diabetes, neurological disorders, and birth defects. Prior to 1990, numerous studies described the abnormal methylation of DNA in tumors and transformed cells. Less frequently investigated, however, were the exogenous and endogenous agents leading to such abnormal methylation. These included genetic variants among rodent strains and the methyl-deficient diets that caused liver cancer. Carcinogens or other promoters that alter methyl metabolism and/or DNA methylation, such as dichlorodiphenyltrichloroethane (DDT), phenobarbital, arsenic, and zinc deficiency, have been studied and have added to the knowledge about the importance of altered methylation on carcinogenesis. By 1990, a chain of causality had been established in experimental carcinogenesis linking dietary methyl deficiency with methyl insufficiency *in vivo*, as well as with the abnormal methylation of DNA and of specific genes. Also, during this period, the diminished activity of the enzyme MTHFR, which is responsible for the actual *de novo* synthesis of methyl groups, was shown to be associated with increased risk of developing atherosclerosis, neurological disorders and birth defects. The exponential rise in studies on methyl metabolism and DNA methylation since then enables us to examine how abnormal methylation processes appear to exert their toxic effects in one disease and how this processes may be applicable to other pathologies.

Dietary Methyl Donor Insufficiency and Human Disease Risk

Insufficiencies in dietary intake of methyl donors and cofactors, such as folate, vitamins B6 and B12, and methionine, have been associated with cancer risk. Much of the early research in this regard focused on the role of cigarette smoke in reducing folate in bronchial tissue; this reduction may influence neoplastic transformation of these cells. Understanding the mechanism of methylation may help explain the increased risk of cancer in smokers. This session of the Workshop focused on dietary methyl donor insufficiency and human disease risk. Topics addressed included a review of methyl donor status and polymorphisms associated with cancer risk, nutritional and genetic inefficiencies in one-carbon metabolism, interactions between methyl donors and cancer risk, and folic acid supplementation and prevention of neural tube defects (NTDs).

Determination of methyl donor status and its relationship to global DNA methylation is important, because the influence of nutrition on gene-specific DNA methylation in humans is not well understood. Radiolabeled methyl incorporation (RMI) and immunohistochemical assay using an antibody to 5-methylcytosine have made it possible to measure methylation at the cellular and tissue level. For example, RMI has been used to determine the level of global DNA methylation in lung tissue, a marker for increased risk of malignancy. It is known that folate and vitamin B12 are involved in the generation of methyl groups for DNA methylation, and that nutritional status with regard to these vitamins may influence global DNA methylation and affect cancer risk. Data

indicate that alterations in global DNA methylation can give rise to epigenetic differences in susceptibility for the development of lung cancer. Finding biomarkers that can reflect the status of global DNA methylation is one focus of current research. For example, a significant association has been observed between the expression of epidermal growth factor receptor (EGFr) -- squamous cell carcinoma of the lung is characterized by increased expression of EGFr -- and global DNA methylation. Future studies also must focus on determining the methylation status of specific genes as well as nutrition-related biomarkers of methylation.

Folate deficiency has long been postulated to play a role in cervical cancer etiology. In a multiethnic community-based case-control study of invasive cervical cancer in 5 U.S. areas, elevated circulating homocysteine was associated with a 2- to 3-fold increase in cervical cancer risk. However, serum and red blood cell folate were only moderately associated with decreased risk, which suggests that elevated homocysteine might be a biomarker of more pervasive problems in one-carbon metabolism than folate deficiency. The contribution of three common polymorphisms in the one-carbon metabolism pathway, two in the MTHFR gene and one in the methionine synthase gene, is being explored. Possible mechanisms whereby inefficient one-carbon metabolism could increase the risk of invasive cervical cancer include 1) impaired DNA synthesis and repair resulting from reduced biosynthesis of purine nucleotides and thymidylate; 2) impaired DNA methylation, such as hypomethylation of proto-oncogene DNA leading to increased expression, selective cell growth, and cell transformation; and 3) integration of HPV at or near an heritable fragile site made susceptible to breakage by inadequate folate.

Folate also has been investigated as a risk factor for colorectal cancer. Animal studies, case-control studies, cohort or prospective studies, and pooled prospective studies have each reported an inverse association between folate status and adenoma and/or colorectal cancer risk. Investigations of *MTHFR* polymorphisms suggest an association with colorectal cancer, which may support the findings for folate. Interactions of folate with other dietary factors that influence methylation, such as alcohol, vitamin B12, and methionine, also appear to influence cancer risk. For example, low folate intake and high alcohol intake increases the risk of adenomas as well as breast cancer. Gender differences are seen in cancer risk at high intakes of folate and methionine. Methionine appears to be more protective in men, and folate appears to be more protective in women.

Folic acid supplementation has had mixed results in the prevention of cancer. However, the dramatic reduction in NTDs in the past three decades is directly related to folic acid supplementation. There has been a significant decrease in spina bifida and anencephaly since folic acid was determined to be related to NTDs. Research related to NTDs has provided a better understanding of folate metabolism, including elucidation of metabolic pathways related to methyl donors and *MTHFR* polymorphisms, as well as the benefit of a threefold increase in folate levels in the U.S. population, in part a result of mandatory food fortification.

Methyl Metabolism and Biochemistry

Studies that characterize methyl metabolism and biochemistry have been undertaken to determine the effects of alterations in methylation pathways by folate, choline, methionine, and vitamins B6 and B12. Deficiencies of these nutrients have been associated with a variety of diseases, including occlusive cardiovascular disease, cognitive impairments, birth defects, alcoholism, diabetes, renal failure, and cancer.

Perturbations in the complex regulation of methylation pathways that maintain one-carbon metabolism and homocysteine homeostasis may promote processes associated with these diseases. This session of the Workshop explored methyl metabolism and biochemistry. Topics included elevated homocysteine levels and DNA hypomethylation, metabolic interactions of alcohol and folate, the effect of abnormal or altered methylation in the brain, pancreas, and liver, and gene-nutrient interactions and DNA methylation.

Research on the role of homocysteine in disease pathogenesis is focused on its role in metabolic pathways associated with methionine cellular methylation cycles, although it has not been determined whether homocysteine is causally involved in disease pathogenesis or is a biomarker for other, more complex mechanisms. Integral to methylation is the methionine metabolite SAM and its conversion to SAH by cellular methyltransferases. This results in methylation of DNA, RNA, proteins, and a variety of other cellular constituents. SAH is further metabolized to homocysteine in a reversible reaction. SAM-dependent methylation has been shown to be central to many biological processes, including metal detoxification and gene regulation by DNA methylation. Elevation in levels of homocysteine results in increased intracellular SAH and consequent product inhibition of DNA methyltransferases. Biochemical studies have shown that plasma homocysteine levels are positively correlated with SAH levels and that total homocysteine and SAH levels are significantly correlated with lymphocyte DNA hypomethylation. In an animal model with cystathionine beta-synthase-deficient (CBS) mice, tissue-specific hypomethylation was more closely related to elevations in intacellular SAH than to decreases in SAM or in the SAM/SAH ratio, and independent decreases in SAM did not induce DNA hypomethylation. In addition, increases in plasma homocysteine were associated with increases in intracellular SAH in all tissues examined except the kidney.

In animals and humans, elevated alcohol intake has been associated with low folate status, reduced folate absorption, and abnormalities in hepatic methionine metabolism, which appear to promote alcoholic liver disease. The metabolic basis for these findings may relate to impaired folate homeostasis, as well as the recognized lower intake of folate seen in binge and heavy drinkers. A study in macaque monkeys reported that there is decreased intestinal absorption, decreased hepatic uptake, and increased urine and fecal excretion of folate with chronic alcohol intake. Studies in micropigs have reported progressive liver damage with chronic alcohol use. In addition, folate-deficiency-related abnormalities in hepatic methionine metabolism were seen with alcohol exposure in monkeys and micropigs. Further studies in micropigs showed that folate deficiency creates alterations in methionine metabolism that accelerate alcoholic liver disease. In addition, sufficient folate appears to protect against alcoholic liver disease.

The affective disturbances associated with folate deficiency and the cognitive defects seen in vitamin B12 deficiency may be related to defective methylation processes in the brain. Folate and vitamin B12 supplementation has improved brain function, suggesting a correlation between deficiencies of these vitamins and neurological abnormalities. Folate and vitamin B12 deficiencies have also been associated with hematological and metabolic abnormalities. For example, folate deficiency is present in 15 -- 38 percent of depressed patients with normal red blood cell counts, and the response to antidepressants correlates positively with folate status. In addition, episodes of depression last longer in patients with lower serum folate levels. Folate metabolites SAM and homocysteine show predictable associations with depression; SAM has an inverse correlation and homocysteine has a direct correlation. *MTHFR* polymorphisms

also appear to be implicated in neurological and neuropathological disorders. Although *MTHFR* deficiency is associated with various disorders, a recent study reported that betaine supplementation alleviated progressive neurological symptoms and normalized the blood levels of methionine and SAM. Homozygosity of the *MTHFR C677T* mutation is significantly increased in schizophrenia, though it does not appear to be a risk factor for cognitive impairment in older persons. Methylatio in the brain is a topic that needs additional research to better understand the role of nutrition in the process of neurological and neuropathological disorders and aging.

MTHFR polymorphisms also have been studied for their effect on methyl metabolism and DNA methylation related to gene-nutrient interactions. DNA methylation in the presence of homozygous and heterozygous genotypes of *MTHFR C677T* was compared by folate status to determine the effect of the interaction. Results indicate that the *TT* genotype is associated with lower folate status, higher homocysteine concentrations, and reduced DNA methylation, when compared with the *CC* or *CT* genotypes. Results have been inconsistent regarding the association of this polymorphism on cancer and cardiovascular risk. This inconsistency is also observed with studies linking hyperhomocysteinemia and disease risk. Understanding the metabolic interactions of this gene and its relationship to folate status may prove to be a promising area of research.

Experimental studies on methyl metabolism in the pancreas provide strong evidence that methyl insufficiency affects pancreatic growth and differentiation, toxicity, and tumor formation. Methionine, choline, and homocysteine have all been studied experimentally to determine their prospective roles in pancreatic growth and differentiation. Pancreatic acinar cells have a high specific requirement for methionine. The pancreatic cells of rats fed methyl-deficient, homocysteine-supplemented diets undergo a dedifferentiation to hepatocytes in chronic feeding studies. Rats and mice fed a diet deficient in choline and containing the methionine antagonist ethionine developed a much greater incidence of acute hemorrhagic pancreatitis than did the corresponding ethionine-treated animals fed the choline-supplemented diet. In hamsters treated with the pancreatic carcinogen N -- nitroso(bisoxopropyl)amine, the latent period for carcinoma induction was dramatically reduced by the intermittent feeding of choline-deficient diet combined with ethionine treatment. One nested case-control study found an inverse relationship between serum folate level and the risk of pancreatic cancer. Altered methylation status has also been studied in diabetics; these studies have shown progressive increases in blood levels of homocysteine and decreases in SAM/SAH ratios during disease progression.

More than 80 percent of methylation reactions and as much as 48 percent of methionine metabolism occurs in the liver. The genes *MAT1A* and *MAT2A* encode the enzyme methionine adenosyltransferase (MAT), which produces SAM and in turn has its expression regulated by SAM. Animal models in *MAT1A*-null mice show increased levels of serum methionine and reduced levels of SAM in the liver, which causes hepatic injury and upregulation of early response genes. This finding indicates that SAM is important, not only as a methyl donor in the methionine pathway, but as a gene regulator responsible for regulating hepatic functions such as regeneration and differentiation.

Mechanisms and Consequences of (Aberrant) Methylation in Physiological Processes

Epigenetic changes can result in changes in gene expression. Mammalian cells have the capacity to modify their genome by covalent addition of a methyl group to the 5'-position of the cytosine ring within the context of the CpG nucleotide. Such DNA

methylation is associated with silencing of gene expression. Both hypermethylation and hypomethylation of the genome have been observed. Epigenetic alterations, specifically region-specific hypermethylation and global hypomethylation, have been implicated either in the etiology or as a consequences of various diseases. This session of the Workshop explored possible methylation-disease associations and hypothesized mechanisms. Topics addressed included DNA methylation and imprinting in development; effect of dietary supplements on DNA methylation and phenotype; and the potential role of DNA methylation in aging, autoimmunity, atherosclerosis, cancer, and the immunodeficiency, centromeric region instability, facial anomalies (ICF) syndrome.

Genomic imprinting refers to a gametic modification of the genome that results in a certain gene expression in the offspring and sets the pattern for postzygotic development. Objectives in this field are to evaluate DNA methylation-dependent versus nonmethylation-dependent mechanisms for genomic imprinting and to assess the role of imprinted genes in development. Imprinting has been studied by direct measurements of allelic expression, analysis of allele-specific DNA methylation and chromatin proteins, characterization of human syndromes involving imprinted genes, and development of mice with deletions of imprinted genes. For example, an imprinted domain on human chromosome 11p15 contains more than 10 imprinted genes/transcripts. The *H19/IGF2* locus in Wilms' tumor maps to this region. Altered imprinting is observed in Wilms' tumor, with *de novo* DNA methylation at *H19* (maternal allele) occurring in 30 percent of tumors. In contrast, DNA methylation does not control allelic expression of *IPL*, an imprinted gene in this domain that controls placental growth.

Early events, including preconception events as well as those from conception to birth, can affect later health. In the "yellow mouse" model, phenotype is epigenetically determined and affects coat color, health, and lifespan. The yellow phenotype (with less DNA methylation) is more susceptible to obesity, cancer, and diabetes and has a shorter lifespan compared to the dark phenotype (with more DNA methylation). Use of this model showed that maternal dietary methyl supplements appeared to increase DNA methylation of a long terminal repeat (LTR) that controls expression of the *agouti* gene (gene associated with coat color) and also increased the number of offspring exhibiting the methylation-dependent phenotype (i.e., lean, healthy animals with a normal lifespan). Such results underscore the need to determine what levels of maternal dietary methyl supplementation are required for humans to ensure optimal health and longevity of offspring.

CpG island methylation can influence cellular physiology through associated chromatin changes and gene silencing and thus can affect disease risk and severity. Epigenetic variation may contribute to a progressive drift of gene expression with aging and may be one of the modulators of acquired, age-related diseases. Studies of methylation in normal human colorectal mucosa show progressive age-related changes at multiple gene loci. Although an important factor, age is not the only factor in epigenetic variation. There is variation in the degree of methylation among individuals of comparable ages, possibly related to genetic and/or environmental factors, including lifestyle.

Data indicate that T-cell DNA becomes hypomethylated with age, and studies in heterozygous *dnmt1* knockout mice suggest that this can contribute to autoimmunity and immune senescence. In one study on human T cells, genome scanning of more than 2,300 CpG islands indicated that, with age, 23 CpG islands became hypermethylated and 6 became hypomethylated. The mechanism that causes the age-dependent changes is unknown, however. Changes in DNA methylation patterns can alter gene

expression patterns, leading to T-cell autoreactivity and autoimmunity. For example, chemical inhibition of T-cell DNA methyltransferases (resulting in hypomethylation) alters expression of more than 100 genes and induces T-cell autoreactivity because of overexpression of adhesion molecules (e.g., LFA-1), and T cells made autoreactive by this mechanism cause a lupus-like disease in vivo. Patients with active lupus and rheumatoid arthritis, both autoimmune diseases, have hypomethylated DNA and decreased DNA methyltransferase activity. Lupus patients also overexpress LFA-1. In lupus, the decreased enzyme activity and consequent DNA hypomethylation may result from defective *ERK* (extracellular signal-regulated kinase) pathway signaling.

Aging and atherosclerosis are considered major risk factors for cardiovascular morbidity and mortality; however, a mechanistic relationship between aging and atherosclerosis has not yet been established. Data indicate that the switch of smooth muscle cells (SMCs) from terminally differentiated to dedifferentiated proliferative phenotype involves DNA methylation and demethylation of a number of important genes. Specifically, the *ER*α gene and the *MCT-3* gene are differentially methylated in proliferating human aortic SMCs *in vitro*, whereas the nucleolin gene is demethylated in such cells. *ER*α gene methylation is associated with aging and atherosclerosis in the cardiovascular system *in vivo*. Further, a correlation exists between DNA methylation and gene expression for *MCT-3* and nucleolin in atherosclerotic coronary arteries. Because *ER*α and nucleolin play important roles in regulating cell proliferation and apoptosis, critical components in the development of atherosclerosis, DNA methylation may serve as a genetic link between aging and atherosclerosis by upregulating susceptibility genes and downregulating protective genes.

Findings from various types of studies support a relationship between diminished folate status and increased risk of carcinogenesis, particularly for colorectal cancer. Epidemiologic studies (adjusted for age) have established that a low intake of vegetables (which are rich in folate) is associated with increased risk for colon cancer, and that a high intake of folate derived from either food or supplements is associated with decreased risk for colon cancer and adenoma (considered to be a precursor for colon cancer). Genetic polymorphisms relevant to folate metabolism (e.g., *MTHFR C677T*) appear to increase colon cancer risk only when folate is deficient. Evidence suggests that folate, which mediates one-carbon transfers, is critical for ensuring the integrity of biological methylation and the nucleic acid synthesis pathways needed to maintain normal DNA metabolism. It has been observed that folate depletion can lead to genomic DNA hypomethylation in humans and hypomethylation of tumor suppressor genes in animal models. Hypomethylation increases the susceptibility of DNA to nuclease attack and misincorporation of uracil, leading to DNA strand breaks and inadequate DNA repair. As a result, DNA integrity is disrupted, resulting in increased DNA damage and alteration of gene expression, which could potentially influence risk for carcinogenesis. Data from animal studies indicate that folate reduces colon cancer risk only if administered at early stages of carcinogenesis and actually may increase risk if administered late in the process.

Estrogens, including phytoestrogens, have been investigated with regard to their potential roles in aging, prostate health, and hormone-related cancers (e.g., prostate, breast). Evidence supports a role for estrogen in loss of imprinting related to cancer; such alterations in imprinting likely result from changes in DNA methylation brought about by estrogens. To illustrate, coumestrol and equol (phytoestrogens) at high doses led to hypermethylation of the *c-ras* oncogene in pancreatic acinar cells, and treatment of neonatal mice with diethylstilbesterol (DES) was linked to hypomethylation of CpG

sites within lactoferrin gene promoter. Various mouse model systems are being used to study the effects of estrogen on DNA methylation. For example, in adult CD-1 mice, *in utero* exposure to high-dose DES increased rDNA methylation patterns, altered reproductive tract development, repressed adult uterine response to estradiol, and increased squamous cell metaplasia of uteri. In a study using ERαKO-TRAMP (TRansgenic Adenocarcinoma of the Mouse Prostate) and ERαWT-TRAMP mice, high concentrations of dietary genistein (a soy phytoestrogen) enhanced prostate tumor progression to the more poorly differentiated adenocarcinoma classification in ERαWT mice, but not in ERαKO mice, suggesting that ERα may be a direct target for estrogenic regulation of prostate tumor progression. Several genes were identified as being differentially methylated in tumor tissue, including the *18S* and *45S* rDNA genes (hyper- and hypomethylation) and *COX17*, a cytochrome oxidase subunit (hypomethylation). Dietary phytoestrogens are available in various commonly consumed vegetables, fruits, and grains, with the highest levels found in soy. However, more research on their definitive roles in aging and cancer is required before specific dietary regimens can be recommended.

The ICF syndrome (immunodeficiency, centromeric region instability, facial anomalies), is the only disease known to be associated with alterations in DNA methyltransferase activity and usually is caused by mutations in *DNMT3b*. This disease is a chromosome instability syndrome in which chromosomal decondensation and rearrangements are targeted specifically to the pericentromeric region of chromosome (Chr) 1 and Chr 16, as determined in mitogen-stimulated lymphocytes and B lymphoblastoid cell lines from these patients. ICF syndrome involves extensive hypomethylation of satellite 2 DNA in centromere-adjacent heterochromatin of Chr1 and Chr 16, where the ICF-specific decondensation is seen. The hypomethylation of the constitutive heterochromatin sequences that is observed in ICF often also is found in diverse types of cancer. Microarray analysis of ICF lymphoblastoid cell lines indicates changes in gene expression for a number of genes, including several that are involved in signal transduction, cell survival, and transcription control.

Research Applications in DNA Methylation

There are four general types of analysis used to characterize DNA methylation with respect to DNA methylation patterns, profiles, and total methylation. These include global analysis of DNA methylation (e.g., methyl acceptance assay, high performance liquid chromatography [HPLC], liquid chromatography/mass spectometry [LC/MS], thin layer chromatography [TLC]); quantitative analysis of single CpG methylation in the DNA pool (e.g., bisulfite sequencing of polymerase chain reaction [PCR], methylation-sensitive, single- nucleotide primer extension [MS-SNuPE], combined bisulfite restriction analysis [COBRA]); semiquantitative analysis of DNA methylation of multiple independent CpG pools (e.g., restriction landmark genome scanning [RLGS], differential methylation hybridization [DMH], epigenomics microarray); and DNA methylation analysis of multiple linked CpGs (e.g., methylation specific PCR [MSP], MethyLight [ML] technology). This session described several of these analytical approaches, along with specific research applications. In addition, the session presented information on the development of a public DNA methylation database (MethDB).

One problem encountered in the molecular analysis of individual gene loci is that DNA methylation patterns are not retained during amplification. This problem can be solved by carrying out methylation-dependent modification before amplification by using either methylation-sensitive restriction enzymes or sodium bisulfite-induced deamination of cytosine. At present, amplification of bisulfite-converted DNA is a widely used

approach. One proposed alternative approach is fluorescence-based, real-time PCR technology (ML technology), a semiautomated technique for analysis of DNA methylation. ML is sensitive and accurate, and ML results have been validated by bisulfite genomic sequencing. For cancer, this technique is applicable to disease detection (e.g., surveillance, biopsy, screening) and treatment (e.g., stage at diagnosis, prognosis, monitoring). For example, in a study of progression of Barrett's-associated esophageal carcinoma, ML was used to determine DNA methylation profiles of tumors and to detect methylation in tissues and fluids. Survival was better for individuals with CpG island hypermethylation of zero to four genes than for those with five to seven genes hypermethylated.

DNA chip (microarray) technologies based on methylation-sensitive restriction include: DMH microarrays, which allow simultaneous analysis of many CpG island loci for one tumor at a time; expressed CpG island sequence tags (ECIST), which allow dual analysis of DNA methylation and gene expression; and "tissue" microarrays, which allow simultaneous analysis of many tumor DNAs for one gene at a time. A DMH microarray panel of 7,776 CpG island tags was applied to detect aberrant DNA methylation in 22 ovarian tumors and 17 breast tumors. In ovarian tumors, hierarchical clustering of hypermethylation patterns of CpG island loci indicated the existence of two clinical subgroups, and the degree of hypermethylation was associated with patients' prognosis. The subgroup with considerable hypermethylation had progressive tumors (no response to chemotherapy) and a disease-free survival time of less than 12 months; the subgroup with little hypermethylation had tumors that responded to chemotherapy and a disease-free survival time of greater than 12 months. In breast tumors, hierarchical clustering of hypermethylation patterns of CpG island loci identified three subgroups of patients and was linked to their hormone-receptor status. The ECIST microarray is useful to differentiate the primary and secondary causes of demethylation and to investigate methylation-silenced genes and the epigenetic activation of downstream genes. It also is useful for examining the efficacy and optimal doses of demethylating agents in cancer treatment. "Tissue" arrays are valuable for *in vivo* validation of novel genes, whose expression is subject to epigenetic control. Also, because they allow simultaneous methylation profiling and direct comparison of multiple tumors, "Tissue" arrays are valuable for rapid assessment of the clinical consequences of epigenetic alterations in cancer.

Epigenomics -- that is, the efficient detection of DNA methylation patterns to determine personal DNA methylation profiles -- combined with statistical analyses and relevant data from epidemiologic and clinical studies, has great promise for developing new and individually tailored approaches for prevention, screening, diagnosis, and treatment of disease. Epigenomic research is an important and rapidly developing area. Digitizing technology applied to epigenetic data is being used to generate Digital Phenotypes™ (Epigenomics AG, Berlin) with potential for facilitating the screening and diagnosis of cancers that are difficult to differentiate clinically. The microarray and mass-spectrometry-based detection platform currently used by Epigenomics allows the analysis of up to 50,000 methylation positions per day. Defining disease more specifically through Digital Phenotypes™ will help to define better treatments. This technology also can be used to monitor the responses of cells to treatments.

A public database for DNA methylation has been established (http://www.methdb.de). This searchable database is valuable because it provides a systematic collection of dispersed literature data, a resource for unpublished experimental data, visualization of methylation patterns and profiles, a basis for computerized data analysis, and matching

of gene expression and methylation data. At the time of this Workshop, the database contained methylation patterns, profiles, and total methylation content data for 41 species, 117 tissues, and 56 phenotypes, from a total of 1,432 experiments. More than half of the data for human tissues refers to DNA methylation in cancer cells. The database was designed to minimize heterogeneity in data and, where possible, to convert data into a comparable format. MethDB has an open structure and can be extended and adapted easily. It is linked to other databases on the Web, including PubMed, GenBank, and the database of imprinted genes; also, a future link with Epigenomics' database may be possible. Efforts will be made to increase the number of MethDB entries relevant to the impact of environmental factors, including diet, on DNA methylation. Future development of MethDB also will include a focus on improved data analysis and submission tools (http://genome.imb-jena.de/methtools/).

Cell and Molecular Biology of DNA Methylation

Although it is now recognized that aberrant DNA methylation is a contributing factor in a number of human diseases, and that hypermethylation of normally unmethylated CpG islands surrounding gene transcription start sites is associated with loss of gene expression, the specific effects of aberrant DNA methylation on the cell and molecular biology processes relevant to human diseases are just beginning to be explored. This session of the Workshop addressed possible mechanisms through which DNA hypermethylation may lead to transcriptional repression--including the roles of chromatin, DNA methyl transferases, and methyl-binding proteins--and related these mechanisms to cancer. In addition, research on the impact of folate deficiency on DNA stability and on certain birth defects was highlighted.

Epigenetic changes, including those associated with DNA hypermethylation, may be viewed as abnormalities of chromatin patterns. Chromatin, a complex of nucleic acids and protein (primarily histone) dispersed in the nucleus that coils and folds to form chromosomes during cell division, is an important part of the cell machinery for gene control. It is now apparent that methylated DNA can serve as a point of origin for the formation of transcriptionally repressive chromatin. For example, in cancer cells, inappropriate targeting of covalent and structural modifications of chromatin to gene promoters that have hypermethylated DNA leads to the silencing of genes required for cell cycle control. Research shows that methylcytosine binding proteins, which have inherent transcriptional silencing activity, can target transcriptional corepressors, histone deacetylases (HDACs), and chromatin remodeling proteins to methylated DNA.

The loss of gene function associated with promoter hypermethylation of tumor suppressor genes -- for example, *APC, BRCA1, MLH1, VHL, p19, E-cadherin, and p16Ink4a* -- is functionally equivalent to the loss of gene function resulting from mutations. For hypermethylated genes in cancer cells, there appears to be a synergistic relationship between hypermethylation and HDAC activity with regard to gene silencing, with hypermethylation having the dominant role. In addition, the DNA methyltransferases (Dnmt1, Dnmt3a, Dnmt3b) can interact with HDACs and transcriptional corepressors to repress transcription. Studies indicate that Dnmt1 works at DNA replication foci to methylate DNA and possibly target newly assembled nucleosome histones for deacetylation. Dnmt3a and Dnmt3b may mediate transcriptional silencing in pericentromeric heterochromatin throughout the cell cycle which may be important in carcinogenesis. Interestingly, in a human colon cancer cell line targeting both *DNMT1* alleles for deletion results in little change in either the overall pattern of methylation or the abnormal hypermethylation of multiple gene promoters. DNA hypermethylation in gene promoter regions--as measured by PCR techniques in

DNA from biological samples such as serum, sputum, bronchial lavage, and urine--has great promise as a molecular marker for cancer detection.

Research on DNA methyltransferases, using mouse knockout models, indicates that the *dnmt1* gene differs from the *dnmt3a* and *dnmt3b* genes in both structure and function. Extensive sequence similarity was found between *dnmt3a* and *dnmt3b*, but little similarity was observed between *dnmt1* and *dnmt3a/dnmt3b*. Dnmt1, the methyltransferase coded for by *dnmt1,* maintained the methylation pattern and had a strong preference for hemimethylated DNA substrates. Inactivation of the *dnmt1* gene resulted in global loss of methylation and embryonic lethality. The methyltransferases dnmt3a and dnmt3b were required for *de novo* methylation activities in embryonic stem cells and during early embryogenesis and were essential for early development. Early embryonic lethality was observed in mice lacking both *dnmt3a* and *dnmt3b* gene activity. Mice lacking only *dnmt3a* activity exhibited growth retardation, short lifespan (3 weeks), a functional obstruction in the small intestine, and hind- limb clasping (a neurological difficulty). Mice lacking only *dnmt3b* activity had hypomethylation of pericentrometric repeats, leading to chromosome instability, and also exhibited growth retardation and early developmental defects. Further, some data indicate that inactivation of *dnmt3a* and *dnmt3b* gene activity may contribute to abnormalities in late development and adult stages in mice.

In other research in mice, conditional mutations were created in *dnmt1* (the maintenance methyltransferase gene) and the methyl-binding protein *MECP2* gene. The inactivation of *dnmt1* leads to global DNA hypomethylation, which results in global gene activation. *Dnmt1* inactivation in embryonic fibroblasts resulted in the derepression of 10 percent of all measurable genes (microarray analysis). The majority of genes were upregulated more than twofold. Induced genes included those with tissue-specific expression *in vivo*, suggesting that DNA methylation is one mechanism for restricting the expression of these genes during development. Interestingly, a small number of genes were downregulated more than twofold. Methyl-binding proteins mediate DNA methylation effects. The methyl-CpG-binding protein 2 (MeCP2) is highly expressed in the postnatal brain, suggesting that methylation-dependent gene silencing may be important in the central nervous system. In humans, mutations in *MeCP2* (an X-linked gene) cause Rett syndrome, a childhood neurological disorder that results in mental retardation in young girls. In this study, *MeCP2*-deficient mice exhibited phenotypes with brain and neuronal changes reminiscent of Rett syndrome; data indicated that *MeCP2* function is essential for both developing and postmitotic neurons.

As noted earlier in this Summary, poor folate status is associated with increased colorectal cancer risk, likely because folate deficiency may impair DNA synthesis and repair and/or alter DNA methylation and gene expression. One study investigated how modulating folate status *in vitro* (human lymphocytes and colon cells), in rats *in vivo* (colon, lymphocytes, and tissues), and in humans (colon cancer case-control trial, human intervention study) affects DNA stability and repair and global DNA methylation. DNA stability was assessed by measuring strand breakage, misincorporated uracil, and oxidative base damage. *In vitro* results indicated that folate deficiency increases uracil misincorporation in human lymphocytes and colon cells, whereas DNA repair of oxidative damage is inhibited. Also, global hypomethylation was observed. In folate-deficient rats, uracil misincorporation was increased only in lymphocytes, and DNA damage increased over time; no changes were observed in DNA methylation in lymphocytes, colon, or liver. In normal, healthy humans, folate supplementation decreased uracil misincorporation in lymphocytes. Colon cancer patients exhibited a

greater frequency of the *MTHFR* homozygous *C677T* variant (but not the *A1298C* variant) than did matched controls. In these patients, DNA methylation status did not vary with polymorphism status. A case-control trial to investigate the potential influence of both the *MTHFR* genotype and dietary folate on DNA stability is under way.

NTDs occur in 1 out of 1,000 live births in the United States. Although periconceptional folic acid supplementation can significantly reduce the risks associated with birth defects such as NTDs, supplementation is not effective in all women, suggesting that genetics may influence risk. Several knockout mouse models have been developed to facilitate the investigation of the gene-nutrient interactions that may underlie susceptibility to the development of birth defects. In these models, either the folate receptor genes *(FOLBP1* and *FOLBP2*) or the reduced folate carrier gene (*RFC1*) have been inactivated by homologous recombination in embryonic stem cells; this allows investigation of the direct effect of a genetically determined folate insufficiency on embryonic development. *FOLBP2* heterozygous matings produced large litters, and no phenotype was associated with the knockout gene. *FOLBP1* heterozygous matings produced small litters, indicating that some process lethal to embryos was associated with this knockout gene. In matings that produced *FOLBP1* and *RFC1* nullizygous embryos, the embryos died *in utero* with significant malformations of the craniofacies, conotruncus, and neural tube. When high doses of a folinic or folic acid supplement were administered throughout gestation of the *FOLBP1* or *RCF1* nullizygous embryos, however, a high percentage of the embryos were rescued (no NTDs). The mouse-model evidence suggests that more than one gene contributes to NTDs and that gene-environment interaction is critically important. The mechanism(s) by which folate protects embryos from NTDs is not yet understood. To date, a genetic survey of approximately 2,000 people has found no coding defects that might be linked to folate and associated birth defects.

Public Health Issues

Generally, scientific research is necessary to lay the groundwork for the development of evidence-based public policy related to specific research areas. Based on research findings, the scientific community can advise policymakers regarding the best practical applications of such findings to achieve optimum public health benefits. This approach also pertains to public guidance regarding folate intake. Numerous questions, however, remain to be answered before folate recommendations can be tailored for individuals. This session of the Workshop presented U.S. data illustrating the effects of folate fortification on folate status, emphasized the importance of understanding the interactions of folate status and metabolism with the genome and their relationship with the disease process before considering individual guidance, discussed the role of biomarkers in determining individual disease risk, and highlighted the difficulty of communicating science-based information and food issues to consumers.

Beginning in 1996, voluntary fortification of enriched cereal and grain products with folic acid was practiced by many manufacturers. The addition of folic acid (140 microgram/100g) to these products became mandatory on January 1, 1998. The objective of this fortification was to increase intake of folic acid by women of childbearing age, thus reducing their risk of a pregnancy affected by NTDs. Subsequent data indicated that fortification with folic acid did, in fact, result in increased serum folate levels. Data from the Framingham Offspring Study cohort showed that, between 1995 -- 1996 (before fortification) and 1997 -- 1998 (after fortification), mean serum folate increased from 4.6 ng/ml to 10.0 ng/ml, and the percentage of individuals with folate less than 3 ng/ml decreased from 22 percent to 1.7 percent. Also, the prevalence of high homocysteine levels (a possible risk factor for vascular disease) decreased by about 50

percent. In another study, Kaiser Permanente data showed that serum folate values increased after 1996, with a median value of 19.0 ng/ml in 1999. National Health and Examination Survey (NHANES) data from 1988 -- 1994 and 1999 showed substantial increases in serum and red blood cell folate concentrations in women of childbearing age. Further, occurrence of NTDs decreased after folic acid fortification; a 19% reduction in NTDs was observed between 1995 -- 1996 (0.38/1,000 live births) and 1998 -- 1999 (0.31/1,000 live births). However, the beneficial effects of folate fortification on risk of NTDs (and also vascular disease) need to be balanced against valid concerns: that high folate may mask vitamin B12 deficiency, and that there is a general lack of data about the safety of continuous high folate intake. (See next paragraph.)

Impairments in folate metabolism--with adverse consequences for genome structure, expression, and stability--can result from folate deficiency *per se* (due to diet, absorption, transport, and/or turnover), secondary nutrient deficiencies (vitamins B6 and B12, iron), and single nucleotide polymorphisms (SNPs) in folate-dependent enzymes. Such impairments will vary among individuals. For example, increased folate turnover rates are associated with cancer, certain pharmaceuticals, and possibly pregnancy. The effects of folate fortification, however, must be carefully monitored. Some data indicate that fortification may increase rates of human spontaneous abortions, presumably by rescuing embryos that would not implant (the embryos then later abort). Further, studies in mutant mouse models, as described earlier in this Summary, have established that high doses of folate can rescue phenotypes with severe genetic lesions to produce viable animals. Extrapolation to humans suggests that continuous exposure to high folate may have unintended consequences for human development and disease susceptibility. Much remains to be learned about the regulation of folate metabolism and corresponding effects on gene expression before it will be feasible to determine individual folate requirements that maximize benefit and minimize potential harm; individual requirements could vary widely as a result of differences in genetic susceptibility, environmental exposures, and health status.

Technological advances, many very costly, have given health service providers the means to more effectively diagnose numerous diseases, including cancer. The use of biomarkers--that is, cellular, biochemical, molecular, or genetic alterations measurable in biological media--is one alternative, cost-effective technological approach to disease detection. Biomarkers can be used to identify individuals at increased risk for disease, resulting from exposure to disease risk factors and/or from certain genetic susceptibilities. Ideal biomarkers should have high sensitivity and specificity as well as high predictive value for disease, be easy to obtain through noninvasive procedures, and be easy to measure with precision and accuracy. Various molecular targets that have potential as risk biomarkers include certain genes, proteins, metabolites (e.g., prostate-specific antigen [PSA]), and hypermethylated DNA. Biomarkers related to cancer risk are a research focus of the National Cancer Institute (NCI). The NCI's Early Detection Research Network (EDRN) is a consortium of more than 32 centers of expertise from academia and industry dedicated to accelerating the development and validation of molecular, genetic, and other biomarkers for assessing individual cancer risk and detecting premalignancy. Future directions in molecular detection and cancer risk assessment include simultaneous measurement of multiple markers and data mining for their interpretation, changes associated with field cancerization (e.g., genomic instability), array technology, and proteomics.

Communicating public health recommendations can be problematic. It is difficult for consumers to understand that science is evolutionary and that one study generally does

not tell the whole story. It is also difficult for consumers to understand that, even when study findings appear to be definitive, broad recommendations based on those findings may not be appropriate for all individuals. Ideally, science communications and health advice should be tailored for specific audiences. Study results frequently are reported to the public without context, which creates confusion. Also, studies may contradict each other, creating even more confusion. Not surprisingly, the attitude of many consumers toward nutrition science and health information is a mixture of confusion, frustration, and apathy and, in fact, the percentage of consumers concerned about nutrition has decreased in the last decade. The majority of consumers think that it is either "somewhat" or "very" likely that "the experts" will have a completely different idea about which foods are healthy and which are not within the next 5 years. To minimize consumer confusion, scientists should strive to help the media understand research and the scientific process and to present research findings appropriately. Each study should be reported without bias and put into the proper perspective, particularly as to how it contributes to the totality of the evidence.

Recommendations for Future Research

The following recommendations, grouped according to the topic areas of the Workshop sessions, include those proposed by the session moderators during the final panel discussion as well as those proposed during the general discussion that followed. The order of listing does not imply either priority or group consensus.

Overview Topics

- Investigate how a hypomethylating environment leads to hypermethylation of genes. How are hypomethylation and hypermethylation related to each other and, what role does folate play?
- Determine what interactions occur between dietary sources of methyl groups in preventing deficiency. Specifically, determine the contributions of betaine-related compounds in providing methyl groups.
- Clarify the tissue specificity of methyl group sources and utilization; different tissues may be dependent on different sources.
- Develop animal models to examine differences among species in folate concentrations in serum, erythrocytes, and other biological samples.
- Examine the possible relevance of findings about methyl metabolism in the liver to data from the National Toxicology Program (NTP). Some chemicals are toxic only at high doses, possibly because they may cause abnormal methyl metabolism, but only at high (toxic) doses. NTP data may help to rationalize hard-to-interpret results from mouse studies.

Dietary Methyl Donor Insufficiency and Human Disease Risk

- Develop a systematic approach to the investigation of single-carbon metabolism that takes individual differences into account, including genetic polymorphisms, as a means to determine who will benefit from elevated folate intakes.
- Develop a model for folate transport to help determine how and at what levels benefit from folate is optimized.
- Investigate the metabolic dynamics of other nutrients and methyl compounds (e.g, betaine, choline) and their relationship with folate.

- Determine the DNA methylation changes that lead to various diseases. For example, which changes lead to NTDs and which changes lead to cancer?
- Establish which genes are modified (and how) by an excess or deficiency of nutrients (all nutrients, not only folate). How does such gene modification vary in individual tissues and over time? This area of research is very complicated, but must be addressed.
- Emphasize research aimed at the validation of biomarkers that can be used to assess disease risk and as surrogate endpoints for clinical disease, including cancer. Biomarker validation is an important crosscutting issue.

Methyl Metabolism and Biochemistry

- Clarify whether SAH is an early biomarker for abnormal DNA methylation. Further, pursue research to determine how SAH relates to abnormal cellular methylation (not only abnormal DNA methylation) in chronic disease.
- Determine how variations in SAH, homocysteine, and glutathione affect the progression of alcoholic liver disease and how supplementation with either folate or SAM might prevent adverse effects.
- Determine if DNA hypomethylation of the brain precedes dementia and cognitive impairment; design and conduct clinical trials to determine if these conditions are reversible by supplementation with folate, vitamin B12, and/or SAM.
- Investigate the pancreas as a useful, sensitive tissue to help understand methyl donor deficiencies and how to prevent various toxicities.
- Conduct investigations into the effects of decreased SAM (with no changes in homocysteine or SAH), using the MATO mouse model (a knockout mouse that spontaneously develops hepatocellular carcinoma), to gain a better understanding of the sensitivity of the liver to toxic insults.
- Further validate the LC/MS method to measure total genomic 5-methylcytosine. Conduct additional studies on the relationship between *MTHFR* polymorphisms and DNA methylation status and the role of this relationship in various diseases.

Mechanisms and Consequences of (Aberrant) Methylation in Physiological Processes

- Continue research to verify the conflict theory of genomic imprinting.
- Continue to study age-related methylation.
- Establish mechanisms important to the role of folate supplementation in methylation and aging.
- Investigate the causes of DNA hypermethylation and hypomethylation and the mechanisms through which it occurs.
- Identify the genes involved in atherosclerosis that are under epigenetic control.
- Explain the mechanisms underlying the deleterious consequences of folate deficiency for colorectal cancer.
- Further explore the pathways involved in methylation and DNA repair in carcinogenesis.

- Investigate and define the mechanisms that link estrogens, including phytoestrogens, to changes in DNA methylation. Also, explore the roles of estrogens/phytoestrogens in aging and carcinogenesis.
- Gain a better understanding of the relationship between DNA hypomethylation, chromatin structure, chromosome stability, and immune function.
- Further explore the mechanisms by which altered DNA methylation patterns contribute to human autoimmunity.

Research Applications in DNA Methylation

- Formalize and standardize data reporting procedures to facilitate uniform reporting of the heterogeneous and diverse data that result from the use of different technologies.
- Establish a common minimal standard for data interchange that would enable independently created local databases for DNA methylation data to communicate with each other. That would make it for instance possible to search all inter-related databases (and possibly mirror sites) from one common query form. This standard should ideally be coordinated with related projects that focus on the integration of different data sources in general (e.g. Object Management Group, see http://www.omg.org/). Alternatively - but less favorable for the moment - establish and maintain a single public DNA methylation database.
- Promote the initiation of a human epigenome project. This should involve a public effort to help determine how the data should be reported, archived, and publicly accessed. Ideally, there could be a different human genome project for every tissue.
- As part of the human epigenome project(s), carry out DNA methylation research corresponding to areas where there are knowledge gaps (e.g., racial groups, aging, nutrition and other environmental factors). Research should be approached in an organized way at the outset so that data will be meaningful in terms of the questions to be answered.
- Emphasize the development of appropriate study designs for DNA methylation research that incorporate population study expertise (e.g., use of sampling techniques, study populations, statistical planning to ensure study power), in conjunction with new analytical techniques. Nutrients must be incorporated into the study designs if a research objective is to obtain information about the potential relationship between nutrients and DNA methylation.
- Encourage collaboration between nutritionists and molecular biologists to conduct the combined epidemiologic-DNA methylation studies necessary to elucidate the relationship between nutrients and DNA methylation.

Cell and Molecular Biology of DNA Methylation

- Emphasize research that will help to explain which factors and mechanisms contribute to changes in DNA methylation. (For example, chromatin and proteins are interdigitated and are associated with methylated DNA to influence gene expression.)
- Clarify the role of nutrients with respect to chromatin and chromatin patterns. It is important to understand the nutritional milieu of the cell, how cell machinery responds to specific nutrients (particularly when cells are injured, affecting methyl

transport), and how chromatin is involved, especially in the context of DNA repair. Research in this area is needed from the cell level to the population level (e.g., influence of genetic polymorphisms).

Public Health Issues

- Emphasize approaches to determine the effects of supplementation with a single nutrient, recognizing that, because of complex nutrient interactions, changing the level of one nutrient may modify the effects of other nutrients.
- Identify and measure (wherever possible) the effects of factors and interactions relevant for intervention trials, recognizing that factors may change over time.
- Evaluate the differences (in terms of benefit and harm) between the population effects and individual effects of folate supplementation. (How do we optimize folate supplementation based on real, currently available data?)
- Design and conduct studies to test the effects of long-term human exposure to folate. Identify the optimum method for current safety monitoring of folate supplementation, considering that possible long-term adverse effects have not yet been established.
- Establish the best way to evaluate the effectiveness of folate supplementation, especially when all the endpoints may not be known.
- Identify and validate biomarkers that can be interpreted in meaningful ways to evaluate the effectiveness of folate supplementation.
- Develop approaches to educate the media and consumers about the evolutionary, self-correcting nature of the scientific process and to improve communication of scientific findings to both the media and consumers. In particular, emphasize putting information into a context that will have applicability for consumers.