The Impact of Nutrition on Differential Methylated Regions of the Genome[1]

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ABSTRACT

Nutrition has always played an important role in health and disease, ranging from common diseases to its likely contribution to the fetal origins of adult disease. However, deciphering the molecular details of this role is much more challenging. The impact of nutrition on the methylome, i.e., DNA methylation, has received particular attention in more recent years. Our understanding of the complexity of the methylome is evolving as efforts to catalog the DNA methylation differences that exist between different tissues and individuals continue. We review selected examples of animal and human studies that provide evidence that, in fact, specific genes and DNA methylation sites are subject to change during development and during a lifetime as a direct response to nutrition. Investigation of the methyl donors folate, choline, and methionine provide the most compelling evidence of a role in mediating DNA methylation changes. Although a number of candidate regions/genes have been identified to date, we are just at the beginning in terms of cataloging so-called nutrient-sensitive methylation variable positions in humans. *Adv. Nutr. 2: 463–471, 2011.*

Introduction

The methylome describes the modifications to DNA that potentially exist within a given genome without changes to the DNA sequence itself. In humans, traditionally these modifications mostly referred to methylation of cytosine residues (5mC),[2] usually in the context of a CpG. However, more recent work indicates that non-CpG methylation (1) and 5-hydroxymethylcytosine (2,3) also form part of the human methylome. Methylation of DNA falls under the umbrella term *epigenetics*, which describes heritable changes to DNA and chromatin that are passed on to daughter cells either mitotically or meiotically. Although histone modifications and their impact on chromatin remodeling are highly relevant and interplay with DNA modifications, they are not the focus of this review [see Choi and Friso (4)].

Research interest in DNA methylation gained momentum in the past 10 years and focused on its effect on gene expression and its association with various disease states, most notably cancer (5). Methylation of DNA in the region of a gene is generally associated with gene expression being switched off, whereas unmethylated DNA is associated with gene expression being switched on. The exact mechanism is not fully elucidated but is believed to involve the interference of methylated cytosine with binding of the RNA polymerase complex and associated transcription factors through an interaction with chromatin (6) (**Fig. 1**). Although this has been the prevailing mechanism, more recent research indicates that DNA methylation is not always associated with transcriptional repression, particularly when it occurs in gene bodies rather than in the promoters of genes (7). Cells use DNA methylation as a mechanism to control gene expression in a number of different ways. The types of DNA methylation changes as they relate to specific functions as we currently understand them range from tissue-specific gene expression to inactivation of imprinted genes, the X-chromosome, and transposable elements. DNA methylation changes have also been associated with a range of disease states including imprinting disorders (8), tumorigenesis (5), neurological disorders (9), cardiovascular disease (10), and autoimmune diseases (11). Apart from definitive pathologically induced changes, evidence to date suggests that there are also regions of the methylome that are subject to change in response to the environment including nutrition. A comprehensive overview of the type of sites that are subject to this is not currently available, but preliminary work on this indicates that it is likely to include regions that silence transposable elements, possibly some imprinted genes and other site types that have yet to be identified. A definitive term has not been assigned to describe these sites that are subject to

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[2] Abbreviations used: CGI, CpG island; DVR, differentially methylated region; 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; IUGR, intrauterine growth restriction; MTHFR, methyltetrahydrofolate reductase; MVP, methylation variable position.

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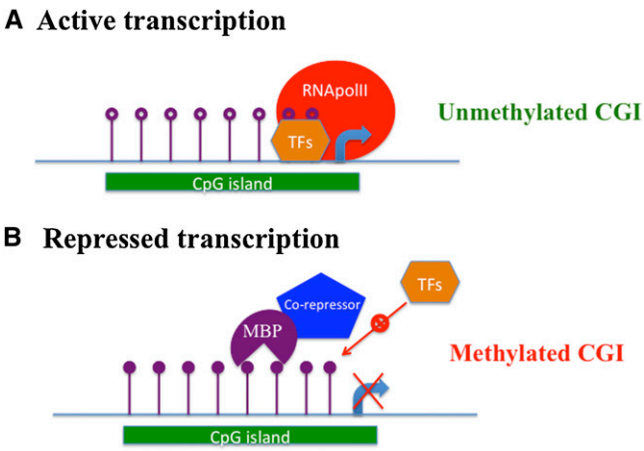

FIGURE 1 The role of CGI methylation in the control of gene transcription. Unmethylated and methylated CGIs in the promoter region of a given gene are shown. Open circle lollipops indicate unmethylated cytosines; closed circle lollipops indicate methylated cytosines. The nucleosomes of chromatin are not shown. *(A)* Unmethylated CGIs are usually in a transcriptionally permissive state in tandem with specific histone modifications. TFs recognize specific DNA motifs in the promoter and facilitate binding of the RNA polymerase II (RNApolII) complex to initiate transcription at the transcription start site (curved arrow). *(B)* Methylated CGIs are associated with a transcriptionally repressed state. This is thought to be due to the binding of MBP, which recognizes methylated cytosines, which subsequently recruit co-repressor complexes that influence the chromatin state via histone deacetylases and ultimately prevent binding by TFs. An alternative mechanism is that methylated cytosines directly prevent binding of TFs. CGI, CpG island; MBP, methyl-binding protein; TFs, transcription factors.

environmental influence, but they have been referred to as metastable alleles or epialleles (12). The Human Epigenome Project (13) uses the term MVP to describe individual DNA methylation sites that show tissue-specific changes or changes in response to a disease state. Others refer to a group of such sites as DMRs (14). Our focus is on those DNA methylation sites that are influenced by nutritional status—so-called nutrient-sensitive MVPs.

Current status of knowledge

The human methylome

Efforts such as the 1000 Genomes Project (15) have provided a comprehensive catalog of the genetic variation that exists between human individuals, but what about the methylome? It is clear that DNA methylation patterns will vary not only among tissues but also among individuals, but the extent and variability of these patterns have yet to be established. The Human Epigenome Project (13) and the NIH Roadmap Epigenomics (16) projects are bids to bridge the gap in our current knowledge of the variation in DNA methylation patterns that exist among individuals. In the same way that single nucleotide polymorphisms provide a catalog of human genetic variation, these projects aim to catalog a variety of epigenetic marks including MVPs, i.e., those DNA methylation sites that vary in different tissues, among individuals, and in disease states.

Extensive human methylome data across a number of different populations and disease states are currently being generated, as alluded to previously. Although such data sets will provide us with a unique insight into human methylome patterns, we rely on recently completed analyses to give us a glimpse of what might be revealed. The first most detailed human methylome, i.e., at single-base-resolution, was published in 2009 by Lister et al. (1). The DNA methylation patterns of human stem cells and fetal fibroblasts were assessed, and a number of interesting patterns were identified that challenged some of the previous assumptions regarding DNA methylation patterns. Previously, most cytosine methylation was thought to occur in the context of CG (or CpG). High-density CG regions are referred to as CGIs, can occur in the promoter regions of genes, and, when they do, are often unmethylated (17). The analysis by Lister et al. (1) revealed that in stem cells, one-fourth of cytosine methylation occurred in a non-CG context and were enriched in the coding regions of genes. Highly expressed genes tended to have a 3-fold higher level of non-CG methylation density than nonexpressed genes. However, this non-CG methylation started to be lost on cellular differentiation and appeared to be a specific feature of stem cells. In human fibroblasts, they noted that reduced methylation levels were associated with decreased transcriptional activity for some genes. Thus, unmethylated cytosines are not always associated with a high level of gene expression, and their impact on transcriptional activity appears to depend on their location within the gene.

5mC was always considered to be the fifth DNA base, but it appears that there is also a sixth base in the form of 5hmC. Although the existence of 5hmC in mammals was originally suggested in 1972 (18), it was only definitively shown in 2009 by Kriaucionis and Heintz (2) and Tahiliani et al. (3). The enzyme that catalyzes the conversion of 5mC to 5hmC was identified as TET1 (3). TET1 is a 2-oxoglutarate and Fe(II)-dependent enzyme that has been shown to bind throughout the genome in embryonic stem cells but particularly at high-density CpG sites in the promoters of genes and within specific genes (19). Modifications of 5hmC showed a pattern similar to that of TET1 binding, i.e., found particularly at high- and intermediate-density CpG sites, which is in contrast to 5mC, which is found predominantly at low-density CpG sites. Similarly to 5mC, however, 5hmC is thought to have a role in transcriptional repression, also mediated through TET1 (19). However, additional roles for 5hmC include the ability to "prime" specific genes for rapid activation when required (20), and it is also thought to have a role in the regulation of DNA methylation patterns (19).

Despite the rapid recent advances in interrogating the methylome, it is clear that understanding the relevance of these methylation marks and how they might vary among tissues and individuals is far from complete. We describe the current evidence demonstrating that nutrition has an impact on 5mC patterns in the following paragraphs, but there are no studies to date that have addressed this specifically at

the 5hmC level. It is likely that nutritional influences are equally relevant for 5hmC given that its formation appears to depend on the presence of 5mC (19). However, the majority of DNA methylation techniques to date are unable to distinguish between 5mC and 5hmC. Therefore, previous studies may need to be reassessed to consider the newly identified sixth DNA base.

Nutrition as a mediator of DNA methylation changes

The identification of verified nutrient-sensitive MVPs is of particular interest because it offers a potential mechanism to explain the observations that relate to the fetal origins of adult disease theory and the role of nutrition in a range of common diseases. The fetal origins of adult disease theory was originally proposed by Barker et al. (21,22) and states that the in utero environment of the embryo can have an impact on the adult disease risk of the offspring. It has also been proposed that some effects are transgenerational, i.e., can be passed on to subsequent generations. This theory was based on initial studies that demonstrated an association between fetal growth impairment and adult cardiovascular disease, diabetes, and insulin resistance (23–27). Fetal undernutrition is believed to be the key mitigating factor, and DNA methylation is one of the mechanisms that may mediate these effects. Additional studies such as the Dutch Hunger Winter found that in utero exposure to famine was associated with increased risks of obesity (men) (28) and schizophrenia (29), increased prevalence of obstructive airways disease (30) and impaired glucose and lipid homeostasis, an increase in coronary heart disease later in life, an increased risk of breast cancer (women), and reduced renal function in a manner that is dependent on gestational age at the time of exposure to famine (31). Evidence to support the fetal origins of adult disease theory has been reviewed extensively elsewhere (32). However, whether it is the changes to the methylome that are mediating these effects remains to be proven.

Apart from in utero exposure, DNA methylation patterns have been shown to change during one's lifetime. The most striking evidence of this was a twin study by Fraga et al. (33) that showed how the methylome of identical twins became more divergent as they aged. They also correlated this with changes in the expression of a range of genes. It is likely that nutrition is one of the environmental influences that have an impact on these methylome changes. Another twin study by Kaminksy et al. (34) suggested that the phenotypic similarity of monozygotic twins is attributed not only to their identical genomes, but also to sharing a similar epigenome compared with dizygotic twins. Further evidence of age-dependent DNA methylation changes has recently been reported for a number of specific genes (35).

It is clear that DNA methylation patterns can change during our development and as we age, but a role in disease development and progression has received particular focus in recent years. The most intensely studied has been changes associated with cancer (5). However, it is difficult to disentangle the sites relevant to nutrition given the complexity and mutational nature of cancer development. Other diseases that exhibit dramatic gene-specific DNA methylation changes include dementia and systemic lupus erythematosus (36).

While we await high-resolution methylome profiles of different tissues from a variety of populations, it is clear that there are regions of the genome that are subject to DNA methylation changes during development, cellular differentiation, or one's lifetime. These may be distinct or overlap with disease-associated DNA methylation changes. The challenge now is to actually identify those nutrient-sensitive MVPs that have relevance to a phenotype. For the remainder of this review, we describe the current evidence of nutrient-sensitive MVPs both in animal models and human studies.

Nutrient-sensitive MVPs

Animal models

The evidence to date suggests that the impact of nutrition on DNA methylation patterns can happen at the level of the organism, probably in a tissue-specific fashion or transgenerationally, i.e., can have an impact on the subsequent progeny. Examples of those nutrients that have been shown to influence DNA methylation patterns are described in the following and are summarized in **Table 1**. Mutant animal models are not included because they complicate the relevance of nutrition on the methylome in these animals.

Methyl donors: folate, methionine, and choline. The folate metabolic pathway consists of a plethora of enzymes and is compartmentalized between the cytoplasm, nucleus, and mitochondria (37). Folate metabolism provides the 1-carbons necessary for a number of reactions involving the synthesis of DNA and in methylation reactions (**Fig. 2**). S-adenosylmethionine is particularly relevant for DNA methylation because it provides the actual methyl group (CH_3) that is added onto cytosine to produce 5mC. S-adenosylhomocysteine is the product formed after donation of the methyl group from S-adenosylmethionine and is an inhibitor of methyltransferases including DNA methyltransferases. Thus, the suggestion that folate supply and other methyl donors such as methionine and choline can influence DNA methylation patterns of an organism has strong biological plausibility. This was elegantly demonstrated in the *agouti* mouse model (38). This mouse model is probably one of the most widely cited animal models in support of the fetal origins of adult disease theory, i.e., transgenerational effects. This study showed how a maternal diet supplemented with methyl-donating vitamins, including folic acid, resulted in hypermethylation of a specific region of the mouse genome, producing an altered coat color phenotype in the mice offspring that were genetically identical (39). Inappropriate expression at the viable yellow *agouti* locus (A^{vy}) due to changes in the methylation status of a cryptic gene promoter led to the variable observable phenotypes. This was one of the first studies that provided experimental evidence in support of DNA methylation as one of the molecular mechanisms mediating maternal diet-related phenotypic

TABLE 1. Summary of selected studies investigating nutrient sensitive DNA methylation sites in animal models[1]

Nutrient	Model	DNA methylation site(s) examined	References
Methyl donor supplementation	Maternal mouse offspring	*Agouti* gene	38–41
		Cdk5 activator binding protein	41
		Axin^{Fu} gene	42
		82 genes including *runx3*	43
Methyl donor deficiency	Maternal sheep offspring	1400 CpG islands	44
	Adult rats	*p53*	45
	Postweaning mouse	*Igf2*	46
	Pregnant mice	*Esr1*, *Igf2*, *Slc39a4CC*	47
	Rat brain	Global methylation	48
Choline deficiency	Maternal mouse offspring	*Cdkn3*, global methylation	49
	Maternal rat offspring	*Dnmt1*, *Ifg2*, global methylation	50
Protein restriction	Adult rats, F2 offspring	PPARα	52, 53
	Maternal mouse offspring	CpG islands including *Lxr*α	54
	Cultured mouse blastocysts	*H19*	55
	Paternal rat offspring	PPARα: CpG island 50kb upstream	58
	Adult mice	Leptin promoter	60
	Maternal rat offspring/leptin injections	PPARα, glucocorticoid receptor	61
High-fat diet	Adult rats	Leptin promoter	59
Genistein	Adult mice	Differential method including ribosomal DNA and desmin-binding fragment	64
Genistein supplementation	Maternal mouse offspring	*Agouti* gene	65

[1] Methyl donors include folate, methionine, and choline.

changes in offspring (39). These DNA methylation changes were also seen to persist in offspring born in the next generation who were not exposed to methyl-donating vitamins in utero (40). Decreased methylation at the A^{vy} locus was also observed in response to the chemical Bisphenol A but was reversed by supplementation of the diet with methyl donors or the phytoestrogen genistein (41). A similar finding was also observed at an additional locus, i.e., the cdk5 activator binding protein (41). Methyl-donating supplementation transgenerational effects have been reported in other animal models including increased methylation at the *Axin*^{Fu} gene in offspring (42). Hollingsworth et al. (43) identified 82 potentially differentially methylated genes in the lung tissue of mouse progeny whose mothers were supplemented with methyl donors compared with controls. Among these, additional convincing evidence identified *Runx3* as being excessively methylated. Apart from rodent supplementation, studies have also considered methyl donor deficiency diets in other animal models. Female sheep were fed a vitamin B-12, folate, and methionine–restricted diet periconceptionally (44). Their offspring were heavier and fatter and had an altered immune response and insulin resistance, and males particularly had elevated blood pressure compared with controls. Methylation of 1400 CGIs was assessed by restriction landmark genome scanning in the fetal liver of the offspring, and 4% of sites showed altered methylation status. Studies investigating the effect of methyl donor deficiency to the individual organism rather than the offspring have also been conducted. The cancer-relevant *p53* gene was demethylated at specific sites in F344 rats fed a diet deficient in the methyl donors choline, methionine, and folic acid for 9 wk. However, it was also noted that some CpG sites were resistant to demethylation (45). Mice subjected to a postweaning methyl donor–deficient diet demonstrated a loss of imprinting of the *Igf2* gene compared with those mice fed the control diet (46). This study also highlights the relevance of the postnatal diet and how it can also influence nutrient-sensitive MVPs. A more recent study (47) assessed the impact of a folate-deficient diet on female mice before and during pregnancy and lactation. DNA methylation patterns of three genes including *Esr1*, *Igf2*, and *Slc39a4CC* were assessed in the dam's blood, liver, and

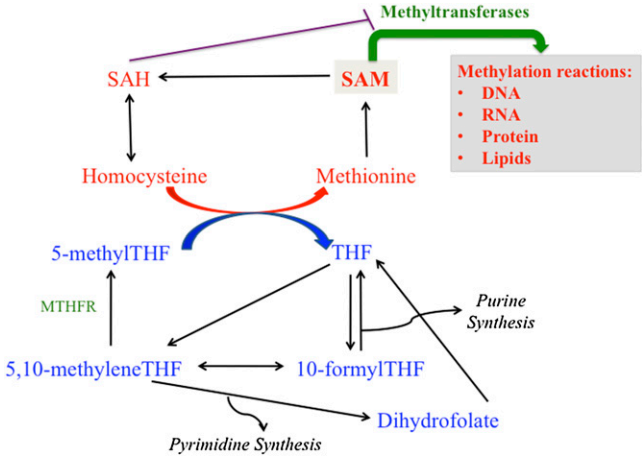

FIGURE 2 A simplified view of cytoplasmic 1C folate metabolism. Not all enzymes are shown. The reactions in blue supply the 1Cs required for DNA synthesis. The reactions in red form part of the folate pathway that produces the methyl donor SAM. A range of methyltransferase enzymes catalyze the transfer of the methyl group (CH_3) from SAM to a number of substrates including DNA, i.e., methylation reactions. SAH is an inhibitor of methyltransferase enzymes. MTHFR, methyltetrahydrofolate reductase enzyme; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine THF, tetrahydrofolate.

kidney. Although the focus of many studies has been on the offspring, this study highlights the relevance of nutrient-sensitive MVPs in pregnant mothers, particularly during a period of high demand for methyl-donating nutrients. DNA methylation changes were observed but were found to be gene and tissue specific. The tissue specificity of DNA methylation changes was also reported in rats fed a diet lacking methionine, choline, and folic acid (48). Global DNA methylation was assessed by HPLC–MS/MS and showed hypermethylation in rat brain and hypomethylation in rat liver compared with controls. However, the biological relevance and sensitivity of measuring global DNA methylation patterns (as opposed to genomewide DNA methylation changes) are not clear. Global DNA methylation by immunohistochemistry (49) and HPLC (50) were also assessed in animal models assessing the transgenerational impact of choline specifically. Dietary choline during embryonic development is thought to have a significant role in memory function in subsequent adults (49,51). A choline-deficient diet was introduced to mouse dams during days 12–17 of pregnancy. Fetal brains were isolated on embryonic day 17, and global DNA methylation was decreased in a specific region of the brain (49). Gene-specific DNA methylation was also assessed by bisulfite sequencing and showed that choline deficiency decreased DNA methylation of the *Cdkn3* gene but had no effect on *Cdkn2b* and *Calb2* (49). A rat study also assessed choline specifically but addressed both supplementation and deficiency. Female rats were fed a diet consisting of varying levels of choline during pregnancy, and the impact of this on DNA methylation of the fetal liver and brain was assessed. Exposure to choline deficiency resulted in reduced methylation of the DNA methyltransferase enzyme Dnmt1 with a corresponding increased level of mRNA. This appeared to lead to increased global DNA methylation and hypermethylation of *Igf2* (50).

These studies illustrate that dietary methyl-donor content can influence DNA methylation patterns, both transgenerationally and at the level of the organism. The propensity to change appears to be gene specific, CpG site specific, and tissue specific. Moreover, changes to global gene expression patterns appear to have little relevance to what is happening at the DNA site–specific level, and, therefore, the relevance of such measurements is questionable. As already mentioned, the link with methyl-donor supply and DNA methylation follows a plausible path, but what about the supply of other nutrients?

Protein and amino acids. Protein restriction is thought to mimic fetal undernutrition, a key factor associated with the fetal origins of adult disease concept. A protein-restricted diet in rats during pregnancy showed loss of DNA methylation in the promoters of glucocorticoid receptor and the PPARα by methyl-sensitive PCR (52). The loss of DNA methylation correlated with an increase in mRNA levels. Interestingly, folic acid supplementation prevented the methylation changes mediated by protein restriction. However, again, gene-specificity is at play here because no change in the promoter of PPARγ, an isoform of PPARα, was observed. A follow-up of this study also showed that the hypomethylation state of the target genes observed in the F1 generation is passed onto the F2 offspring, even when the F1 offspring were fed a normal diet (53). Maternal protein restriction was also observed to affect DNA methylation of a range of genes by CGI microarray analysis in fetal livers shortly after birth (54). Further analysis of one of these genes, i.e., liver-X-receptor alpha (*Lxr*α), showed hypermethylation in protein-restricted pups that correlated with a drop in mRNA levels. Loss of imprinting has also been observed in cultured mouse blastocysts at the maternally expressed *H19* gene (55). Demethylation at the normally hypermethylated paternal allele was dependent on the culture medium used. Medium containing extra amino acids did not display loss of imprinting, highlighting the relevance of the nutritional environment for the preservation of imprinting patterns. Again, gene specificity is observed because another imprinted gene, *Snrpn*, did not display loss of imprinting in either medium. In addition to maternal transgenerational nutritional effects, evidence of paternal effects has also been shown (56,57), which highlights the importance of considering alternative mechanisms beyond maternal effects. Offspring livers of male rats on a low protein-diet showed a significant increase in DNA methylation at an intragenic CGI, $\sim$50 kb upstream of the *PPAR*α gene by bisulfite sequencing. Because the global methylation levels in low-protein and control offspring were similar, this further demonstrates the relevance of gene specificity (58).

Fat. Leptin is an adipokine that is involved in the regulation of body weight and food intake. A recent study describes how a specific CpG site within the leptin promoter showed significant methylation differences in rats fed a high-fat diet compared with controls. The change in methylation was associated with circulating leptin levels (59). Changes in the leptin promoter, i.e., the removal of methyl groups at CpG sites within the promoter, was also shown in mice fed a low-protein diet (60). The impact of leptin on DNA methylation was further investigated by Gluckman et al. (61). Adult rats that were previously injected with leptin after birth to well-nourished mothers demonstrated a differential methylation change in PPARα and glucocorticoid receptor compared with those born to undernourished mothers. This article highlights how maternal diet history can influence subsequent neonatal and adult cellular responses.

Genistein. Soy products are known to reduce the risk of cardiovascular disease and carcinogenesis (62,63). Genistein is a soy phytoestrogen that may mediate these risk reductions and was investigated for an impact on DNA methylation. Day et al. (64) used differential methylation hybridization arrays to investigate DNA methylation in mice on a genistein diet. Liver, brain, and prostate tissues were examined, but only changes in the prostate were detected using this technique. These included three novel sequences, ribosomal DNA, and a desmin-binding fragment. However, confirmation

of these changes with an additional technique was not performed, and, therefore, their relevance is unclear. Genistein supplementation did, however, increase methylation at the A^{vy} locus in the *agouti* mouse model offspring (65) in a fashion similar to methyl donor supplementation, as described previously.

Animal models have provided substantial evidence that fetal undernutrition, or indeed overnutrition, can influence DNA methylation patterns of an individual or their offspring. Are humans susceptible to the same types of changes in response to nutritional status? It is much more challenging to address this in humans.

Human studies

Although there is a wealth of literature supporting the role of nutrition in health and disease including the fetal origins of human disease concept (32), the identification of nutrient-sensitive MVPs in humans has only been a recent focus of attention. The Dutch Hunger Winter from 1944 to 1945 provided an excellent opportunity to investigate this further. Food restrictions were in place in the western part of the Netherlands toward the end of World War II. Despite this, medical records and registries were maintained, which allowed prenatally exposed individuals to be traced. Heijmans et al. (66) showed that prenatally exposed individuals displayed significantly less DNA methylation at the imprinted *IGF2* locus compared with their unexposed, same-sex siblings 60 y later. This appeared to be an early developmental effect because individuals exposed during late gestation showed no effect. The susceptibility of the *IGF2* locus to in utero exposure in humans correlates with similar findings in animal models already described. Additional loci for this cohort of samples were examined in a subsequent study that found that *INSIGF* showed decreased methylation, whereas *IL10*, *LEP*, *ABCA1*, *GNASAS*, and *MEG3* showed increased methylation in individuals exposed prenatally to the famine (67). Late gestational exposure showed DNA methylation changes in just *GNASAS* and *LEP* in men only. Thus, nutrition-induced changes appeared to be particularly relevant but not limited to the periconceptional period, showed both increased and decreased methylation, and displayed some sex specificity. Apart from the Dutch Hunger Winter samples, a number of both observational and intervention studies have been conducted in humans, focusing for the most part on methyl donors, particularly folate. We did not include those studies involving cancer tissues because epigenetic dysregulation is well documented as contributing to tumorigenesis and may cloud the role of nutrients in mediating DNA methylation changes. Friso et al. (68) reported a correlation between folate status and global DNA methylation levels in peripheral blood mononuclear DNA measured by HPLC/MS. Again, the relevance of measuring global DNA methylation without site-specific information is not clear, but the authors did report the interesting observation that individuals who were *MTHFR* 677 TT homozygous had a significantly lower level of global DNA methylation compared with 677 CC individuals. The *MTHFR* gene encodes a key enzyme in the folate metabolic pathway (**Fig. 2**), i.e., 5,10-methyltetrahydrofolate reductase. The *MTHFR* 677C > T polymorphism is a well-accepted, disease-associated variant that is thermolabile and converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate at a reduced rate, particularly when the folate status is low (69,70). Thus, the supply of methyl groups may be compromised in *MTHFR* 677 TT individuals, and this appears to correlate with global DNA methylation patterns. Additional observational studies focusing on folic acid include an examination of promoter methylation of estrogen receptor α and *MLH1* (71). This study found no correlation between serum and red cell folate and DNA methylation but did find a correlation between vitamin B-12 and estrogen receptor α methylation in colon tissue. Steegers-Theunissen et al. (72) found a 4.5% increase in the DNA methylation of *IGF2* DMR in the children of mothers who consumed 400 μg of folic acid periconceptionally compared with those who had not. Folic acid intervention studies of postmenopausal women showed decreased global DNA methylation in women consuming a low-folate diet measured by incorporation of a tritiated methyl group using SssI methyltransferase (73,74). However, one study reported recovery of DNA methylation in folate-replete subjects (73) but no change in another (74). Again, we question the validity of measuring global DNA methylation levels as opposed to a genome-wide analysis. A more recent report by Fryer et al. (75) applied the Illumina Infinium Methylation 27K BeadArray system to human cord blood samples in which folate and homocysteine levels were also measured. Homocysteine is inversely correlated with folate levels (**Fig. 2**). This allows analysis of 27,578 CpG loci associated with 14,496 genes and is a large step toward a genomewide analysis. Similar to previous findings [reviewed by Deaton and Bird (17)], the general pattern observed was that the majority of CpGs within CGIs were found to be hypomethylated, whereas those outside CGIs were methylated at a mid to high level. They specifically identified that DNA methylation of 12 genes (*EIF2C3*, *ZBTB11*, *BDH2*, *ZNF187*, *RUNX1T1*, *C9orf64*, *PDE2A*, *MGC33486*, *AMN*, *ZPBP2*, *FBN3*, *PVRL2*) directly correlated with homocysteine levels, whereas the methylation of 5 genes (*ATP5F1*, *CYP26C1*, *FSTL3*, *MDS032*, *BMX*) displayed an inverse correlation with homocysteine levels. Because cord blood samples were taken at term, it supports late gestation as also representing a window of opportunity for nutrition-induced DNA methylation changes. However, the methylation patterns observed by the Illumina Infinium Methylation 27K beadchip were not confirmed by another method, and, therefore, these genes require further validation. The limited number of human studies described here does implicate folate status and its associated metabolites in influencing DNA methylation patterns both in utero and in adults. Assessment of other nutrients has been limited. An intervention study of soy isoflavones in 34 healthy premenopausal women showed that the promoter region of *RARβ2* and *CCND2* in breast tissue showed hypermethylation post-treatment but no effect on *p16*, *RASSF1A*, or *ER* (76). The relevance of

TABLE 2. Summary of selected studies investigating nutrient-sensitive DNA methylation sites in humans[1]

Nutrient	Study type	DNA methylation site(s) examined	References
Calorie restriction	Maternal offspring	*IGF2*, *INSIGF* in adults	66, 67
		IL10, *LEP*, *ABCA1*, *GNASAS*, *MEG3*	67
Folate status	Adults/MTHFR 677C > T genotype	Global methylation	68
Folate, vitamin B-12 status	Adults: colon	*ERα*, *MLH1*	71
Folic acid consumption	Maternal offspring	*IGF2*	72
Folic acid intervention	Postmenopausal women	Global methylation	73,74
Folate/homocysteine status	Cord blood	14,496 genes	75
Soy isoflavone intervention	Premenopausal women	*RARβ2*, *CCND2*, *p16*, *RASSF1A*, *ER*	76

[1] MTHFR, methyltetrahydrofolate reductase.

gene specificity is again highlighted here. DNA methylation patterns and IUGR were considered using a microarray DNA methylation assay known as HELP (HpaII tiny fragment Enrichment by ligation Mediated PCR) (77). Although specific nutrients were not examined, nutrient status has been implicated as a contributing factor to IUGR (77). A modest change of 6% DNA methylation difference was observed consistently in *HNF4A*, *ATGS*, and *TADA3L* in the cord blood of IUGR neonates compared with controls. These genes were previously linked with type 2 diabetes.

The identification of nutrient-sensitive MVPs in humans is limited by sample availability and ethical issues. Despite this, a number of observational and intervention studies found DNA methylation changes in response to nutrient status, particularly in response to folate status (summarized in **Table 2**). The more relevant studies examined gene-specific DNA methylation patterns (66,67,71,72,76) or a narrow genomewide approach (75). Candidate methylation sites have emerged, but we are at the beginning in terms of deciphering the human methylome and identifying those nutrient-relevant DNA methylation sites.

Conclusions

Efforts in the past 10 y provide us with a glimpse of the complexity of the human methylome. We now know that the simple association of DNA methylation and repressed gene expression is not always the case. Mammalian DNA methylation can occur outside the CG context and within the coding regions of genes. Methylcytosine can also be converted to hydroxymethylcytosine and is recognized by a specific set of molecules that are just starting to be understood. Data just published (78) have finally begun to shed light on how demethylation of DNA occurs, i.e., by conversion of 5mC and 5hmC to 5-carboxylcytosine with subsequent processing by thymine-DNA glycosylase. Cataloging the methylomic differences between tissues and individuals has just begun, but what is clear is that nutrition has a distinct role to play in mediating DNA methylation changes during development or one's lifetime. Currently there is no comprehensive list of which sites are nutrient sensitive, but studies to date, some which are described here, provide potential candidates. These studies highlighted how it is not simply about methyl group supply because both supplementation and deficiency studies observe both increases and decreases in DNA methylation. The pattern that is emerging so far is that nutrient-mediated DNA methylation changes are gene specific, site specific, tissue specific, and age specific. In light of this, it is much more meaningful to examine specific, known regions of the genome or, indeed, a genomewide screen rather than measuring global DNA methylation patterns that have limited relevance. The technology is now available to do this, and the identification of nutrient-sensitive MVPs is likely to explode in the next few years.

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