THE EFFECTS OF MATERNAL FOLIC ACID SUPPLEMENTATION PROVIDED IN UTERO AND DURING LACTATION ON GENOMIC DNA METHYLATION AND GLOBAL GENE EXPRESSION IN THE OFFSPRING

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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ABSTRACT

The effects of high maternal folate status on the offspring are unknown; however, early life exposure to environmental stimuli, including dietary factors, is thought to influence the risk of developing chronic disease. An animal study was performed to determine the effect of maternal folic acid supplementation during pregnancy and lactation on DNA methylation and global gene expression patterns in the offspring. Supplementation of the maternal diet with folic acid (5, 8, and 25 mg/kg diet) induced global DNA hypomethylation in the liver of juvenile and adult offspring, and altered the expression of the imprinted *H19* gene and genes involved in lipid metabolism, coagulation, and iron transport and homeostasis. Our data suggest that intrauterine and early postnatal periods are susceptible to changes in DNA methylation and gene expression in response to high levels of folic acid, which may be associated with health status and disease later in life.

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LIST OF ABBREVIATIONS

ACACA	Acetyl CoA carboxylase-1
ALL	Acute lymphoblastic leukaemia
AOM	Azoxymethane
APC	Adenomatous polyposis coli
APOB	Apolipoprotein B
APOM	Apolipoprotein M
ATP	Adenosine-5'-triphosphate
BDR	Basal dietary requirement
BPA	Bisphenol A
BWS	Beckwith-Wiedemann syndrome
CACNA1G	Calcium channel
CD36	CD36 molecule
CDH1	E-cadherin
CDH13	H-cadherin
CIMP	CpG island methylator phenotype
C-MYC	V-myc myelocytomatosis viral oncogene homolog
CNNM1	Cyclin M1
CpG	Cytosine-guanine dinucleotide
CPT1A	Carnitine palmitoyltransferase-1A
CRABP1	Cellular retinoic acid binding protein 1
CRC	Colorectal cancer
CTCF	Zinc-finger protein CCCTC-binding factor
CTNNB1	β-catenin
DFEs	Dietary folate equivalents
DHFR	Dihydrofolate reductase
DMBA	Dimethylbenzanthracene
DMH	Dimethylhydrazine
DMR	Differentially methylated region
DNMT	DNA methyltransferase
DRIs	Dietary reference intakes
dTMP	Deoxythymidine-5-monophosphate
dUMP	Deoxyuridine-5-monophosphate
EAR	Estimated average requirement
ER	Estrogen receptor
FBP	Folate binding protein
FPGS	Folylpolyglutamate synthetase
FR	Folate receptor
G6PC	Glucose-6-phosphatase
GCP-II	Glutamate carboxypeptidase II
GGH	γ-glutamyl hydrolase
GR	Glucocorticoid receptor
HAMP	Hepcidin antimicrobial peptide
HAMP2	Hepcidin antimicrobial peptide 2
HDL	High-density lipoproteins

HP	Haptoglobin
IAP	Intracisternal A particle
ICC	Intracluster correlation coefficient
ICM	Inner cell mass
ICR	Imprinting control region
IGF2	Insulin-like growth factor 2
IGF2BP3	Insulin-like growth factor 2 mRNA binding protein 3
INS1	Insulin 1
KNG2	Kininogen 2
LDL	Low-density lipoproteins
LINE-1	Long interspersed nuclear element-1
LPL	Lipoprotein lipase
MAPK4	Mitogen-activated protein kinase 4
MBD	DNA-methyl-binding domain proteins
MDM2	MDM2 P53 binding protein homolog
MINT	Amyloid beta A4 precursor protein-binding
MLH1	MutL homolog 1
MTHFR	Methylenetetrahydrofolate reductase
MTR	Methionine synthase
MTRR	Methionine synthase reductase
MUP	Major urinary protein
NADPH	Nicotinamide adenine dinucleotide phosphate
NEUROG1	Neurogenin 1
NHANES	National Health and Nutrition Examination Survey
NK	Natural killer cell
NTDs	Neural tube defects
O^6-MGMT	O-6-methylguanine-DNA methyltransferase
OD	Optical density
p16	Cyclin-dependent kinase inhibitor 2A
p53	Tumor protein 53
PABA	Para-aminobenzoic acid
PCFT	Proton-coupled folate transporter
PCK1	Phosphoenolpyruvate carboxykinase
PCR	Polymerase chain reaction
PHLDA2	Pleckstrin homology-like domain, family A, member 2
PLG	Plasminogen
PPAR	Peroxisome proliferator–activated receptor
PRODH1	Proline dehydrogenase (oxidase) 1
RBC	Red blood cell
RDA	Recommended dietary allowance
RFC	Reduced folate carrier
RIN	RNA Integrity Number
RLGS	Restriction landmark genomic scanning
RUNX3	Run-related transcription factor 3
SAH	*S*-adenosylhomocysteine
SAM	*S*-adenosylmethionine

SERPIND1	Serpin peptidase inhibitor, clade D member 1
SERPINF2	Serine (or cysteine) peptidase inhibitor, clade F, member 2
SHMT	Serine hydroxymethyltransferase
SMAD-4	SMAD family member 4
SOCS1	Suppressor of cytokine signalling 1
SOGC	Society of Obstetricians and Gynaecologists of Canada
SPP1	Secreted phosphoprotein 1
SREBP-1c	Sterol regulatory element binding transcription factor 1
TCE	Trichloroethylene
TE	Trophectoderm
THF	Tetrahydrofolate
TRF	Transferrin
TRFC	Transferrin receptor
TS	Thymidylate synthase
UL	Tolerable upper intake level
ZBTB16	Zinc finger and BTB domain containing 16

Chapter 1: INTRODUCTION

Folate is a water-soluble B vitamin found naturally in a variety of foods including green leafy vegetables, and encompasses a family of compounds with similar chemical structures and nutritional properties [1]. Folate facilitates the transfer of one-carbon moieties in biochemical pathways involved in DNA synthesis and methylation reactions, and deficiency of the vitamin has been shown to play an important role in the pathogenesis of various disorders including anemia, atherosclerosis, adverse pregnancy outcomes, and cancer [2].

The wealth of evidence from observational studies and intervention trials demonstrating the protective effect of folic acid, the synthetic form of the vitamin, on the risk of neural tube defects (NTDs) led to the implementation of mandatory folic acid fortification in both the United States and Canada in 1998 [3, 4]. Reducing the rate of NTDs, the primary goal of the fortification program, has been deemed successful as studies suggest an approximate 15-50% decrease in the prevalence and incidence of NTDs in both the United States and Canada [5-8]. In addition, folic acid fortification has significantly improved folate status, as a dramatic increase in blood folate measurements and folate intake and a substantial decrease in plasma homocysteine concentrations (an inverse indicator of folate status) have been observed across all gender and age groups following the initiation of the program [9].

In women of childbearing age in particular, median serum folate levels increased greater than two-fold, and red blood cell (RBC) folate concentrations drastically increased following the implementation of mandatory folic acid fortification [10]. In addition to folic acid fortification, the widespread consumption of multivitamins containing folic acid and

current recommendations advising women of childbearing age to consume a prenatal supplement containing folic acid to reduce the rate of NTDs have significantly contributed to the substantial improvement of folate status in women of childbearing age [11, 12]. Currently, it is recommended that women of childbearing age consume a minimum of 400 μg folic acid daily from food and/or supplements, and many prenatal multivitamins contain 1 mg of folic acid [13]. Recently, however, the Society of Obstetricians and Gynaecologists of Canada (SOGC) and Motherisk have jointly published new guidelines recommending that certain women of childbearing age, including those with a history of poor compliance with medications or specific health conditions, consume a daily supplement containing 5 mg of folic acid [12].

A high maternal folate intake not only exposes the pregnant woman to elevated levels of folate, but also the developing offspring. At present, the effects of intrauterine and early postnatal exposure to supplemental levels of folic acid on health outcomes in the offspring other than NTDs are largely unknown. However, emerging evidence suggests that exposure to environmental stimuli, including dietary factors, at the earliest stages of life may influence the risk of developing disease later in life in the offspring, a concept known as 'fetal programming' [14]. Several recent studies suggest that increased exposure to folate early in life may increase the risk of adverse health outcomes in the offspring, including obesity, insulin resistance, and asthma [15-17].

DNA methylation, an epigenetic modification of gene expression, has been suggested to be one possible mechanism in which dietary factors such as folate may induce fetal programming. Folate plays an essential role in the provision of *S*-adenosylmethionine (SAM), the universal methyl donor for most biological methylation reactions including that

of DNA. Both folate deficiency and supplementation in the young, during adulthood, and in the elderly, have been shown to modify DNA methylation patterns [18, 19]. During embryogenesis, DNA methylation patterns are reprogrammed as the epigenome undergoes widespread demethylation resulting in the erasure of significant parts of the parental DNA methylation, followed by *de novo* methylation in which new DNA methylation patterns are established [20]. Therefore, the epigenome may be highly sensitive to environmental influences during these earliest stages of life. Waterland and Jirtle demonstrated that maternal supplementation with high levels of methyl donors, including folic acid, can permanently affect the offspring's DNA methylation at epigenetically susceptible loci [21]. Furthermore, numerous studies have since shown that alterations in maternal nutrition throughout pregnancy and/or the early postnatal period can modulate DNA methylation in the offspring [21-24].

In utero and/or early postnatal exposure to high levels of folate alone may also influence DNA methylation patterns in the developing offspring. A high maternal folate status could alter the methylation of cytosine-guanine dinucleotide (CpG) sites located in the bulk of the genome including exons, noncoding regions, and repeat DNA sites. In addition, a high maternal folate status could modulate the methylation status of CpG islands spanning the 5' end of human genes including the promoter region, untranslated region, and exon 1, which could lead to persistent changes in offspring gene expression. The functional ramifications of altered global, gene- and site-specific DNA methylation resulting from high intrauterine and early postnatal folate exposure may ultimately influence the health of the offspring.

The induction of changes to the phenotype of the offspring, which may influence health outcomes later in life, ultimately implies stable changes in gene expression. At present, numerous *in vitro*, animal, and human studies have demonstrated that both folate deficiency and supplementation in the young, during adulthood, and in the elderly are capable of modulating the expression of various genes [25-29]. Thus, maternal folic acid supplementation may also alter the expression of genes in the offspring. Several recent animal studies have shown that maternal folic acid supplementation in combination with a protein restricted diet can alter gene expression in the offspring [30-32].

To date, no studies have investigated the effect of increasing levels of isolated maternal folic acid supplementation, including very high doses paralleling the recent SOGC and Motherisk recommendations in humans, throughout the *in utero* and early postnatal periods on global DNA methylation and genome-wide gene expression patterns in the offspring in a mouse model. Thus, the main objective of the present study was to determine whether varying levels of maternal folic acid supplementation throughout pregnancy and lactation would modulate global DNA methylation and gene expression patterns in the offspring.

Chapter 2: LITERATURE REVIEW

2.1 Folate

2.1.1 Chemistry and Properties

Folate is a water-soluble B vitamin, that encompasses all B-vitamin compounds exhibiting similar chemical structures and nutritional properties to the parent structure of folic acid (pteroylmonoglutamate, PteGlu) [1]. The chemical structure of folic acid consists of three distinct components: para-aminobenzoic acid (PABA) is attached at one end to a 2-amino-4-hydroxy-pteridine (pteridin) moiety via a methylene group, and at the other end to one L-glutamic acid (glutamate) residue via a peptide bond **(Figure 2.1)** [33].

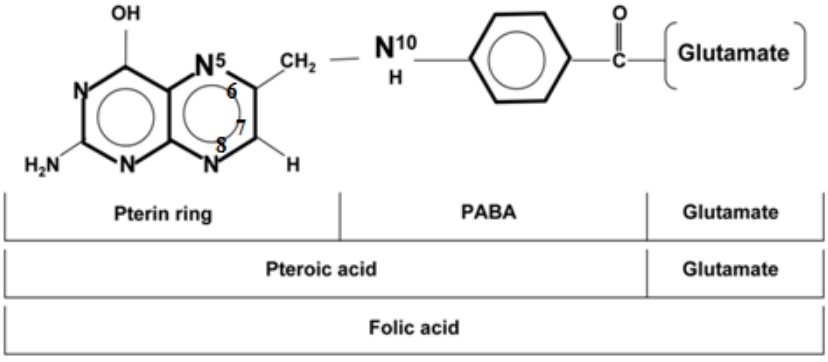

Figure 2.1 Chemical structure of folic acid (pteroylmonoglutamate, PteGlu). Folic acid consists of three distinct components: the pterin (or pteridine) ring, which is conjugated to PABA (para-aminobenzoic acid) by a methylene bridge, which is in turn joined to a glutamic acid (glutamate) residue via a peptide bond. Modified from [2].

Folic acid is the fully oxidized, monoglutamyl form of folate and becomes biologically active following the reduction of the pyrazine ring at the 5, 6, 7, and 8 positions of the pterin moiety to tetrahydrofolate (THF) [33]. Naturally occurring folates found in foods differ from the oxidized folic acid in the oxidation state of the pteridine, and are typically reduced derivatives. Natural folates exist in a variety of forms varying according to the number of glutamic acid residues (polyglutamate) bound to PABA. In addition, natural forms of folate differ according to the one-carbon units linked at the N-5 and/or N-10 positions of THF **(Table 2.1)** [1]. The stability of reduced folates depends on the one-carbon substitution; however, reduced folates are typically less stable than folic acid [33].

Table 2.1 One-carbon substitution of tetrahydrofolates

Folate Derivative	One-Carbon Substitution
5-FormylTHF	-CHO
5, 10-MethenylTHF	=CH-
5, 10-MethyleneTHF	$=CH_2-$
5-MethylTHF	$-CH_3$
10-FormylTHF	-CHO
5-ForminoTHF	-CH=NH

*THF=tetrahydrofolate

2.1.2 Sources and Bioavailability

Mammals cannot synthesize folate *de novo* as they lack the enzyme responsible for coupling the pteridin moiety to PABA [33]. On the other hand, bacteria within the human intestine are capable of synthesizing folate, although the amount incorporated into the host's tissues has been suggested to be minimal [34, 35]. However, a recent study has shown that physiological doses of natural folate are absorbed across the intact colon in humans [36].

Mammals can obtain folate from exogenous sources such as food and supplements. Folate is found naturally in various foods such as green leafy vegetables (spinach, turnip greens, arugula), asparagus, broccoli, cauliflower, and citrus fruits [1]. Meat is not a good source of folate, with the exception of liver [37]. Due to the fact natural reduced folates are more labile than oxidized folic acid, large losses can occur during food processing, preparation, and storage; therefore, the concentration of natural folate in raw food is typically greater than in cooked foods [1]. Over 150 forms of folate in foods have been identified; however, the main forms are 5-methylTHF and 10-formylTHF [1].

Folic acid is a synthetic form of folate. Due to its greater stability and lower cost, it is typically the form used commercially in vitamin supplements (as folic acid alone or as a component of a multivitamin) and in enriched and fortified food products [38]. In addition, a stable, reduced supplemental form of folate, L-5-methylTHF, is now available in the United States and European Union for use in dietary supplements and medical foods [39].

The bioavailability of folate refers to the proportion of ingested folate that is absorbed and available for metabolic process or storage [40]. Folic acid from supplements is almost 100 percent bioavailable when consumed under fasting conditions [41]. In addition, the bioavailability of L-5-methylTHF is comparable to that of folic acid [42]. The bioavailability of folic acid consumed with food is slightly lower and is estimated to be 85 percent [41]. The bioavailability of folate found naturally in foods is incomplete when compared with the synthetic form of folic acid; however, the extent to which bioavailability is reduced has been shown to vary, ranging from 10 to 98% [40]. In a recent study, it has been suggested that the bioavailability of folates from fruit, vegetables, and liver is approximately 80 percent of that of folic acid [43].

The absorption of naturally occurring folate may be hindered by several factors, including partial release from the food matrix, destruction within the gastrointestinal tract, and incomplete hydrolysis of glutamates [44]. In addition, the bioavailability of both naturally occurring folate and folic acid may be hindered by a variety of post-absorptive factors specific to the individual, including sex, ethnicity/race, and pool sizes of folate as well as other relevant nutrients.

Dietary folate equivalents (DFEs) were introduced in 1998 in order to account for bioavailability differences between naturally occurring food folates and synthetic folic acid, and are typically used when estimating dietary intake recommendations [45]. Since folic acid consumed with food is 85 percent bioavailable, while naturally occurring food folate was originally estimated to be only 50 percent bioavailable, folic acid consumed with food is estimated to be 1.7 (85/50) times more available than food folate [41]. For this reason, total dietary folate intake is expressed as: µg of food folate + (1.7 x µg of folic acid).

2.1.3 Folate Metabolism

Although folate absorption can occur throughout the intestine, it appears to be most efficient in the proximal part of the small intestine, particularly in the jejunum [1]. Natural folates from food typically exist as polyglutamates and do not readily cross the cell membrane; therefore, prior to absorption across the intestinal mucosa, polyglutamates must first be hydrolyzed to the monoglutamate form by the zinc-dependent intestinal brush border enzyme glutamate carboxypeptidase II (GCP-II) **(Figure 2.2)** [33]. GCP-II has a low pH optimum, consistent with the low pH environment in the proximal jejunum at the surface of the intestinal mucosal cells [46]. In contrast to natural folates in food, hydrolysis is not

required for absorption of folic acid as it contains one glutamate residue. Following the hydrolysis of polyglutamate forms of folate, reduced monoglutamyl folates are absorbed.

At present, the biological mechanism by which folate crosses the mucosal cell and is released across the basolateral membrane into the portal circulation has not been clearly elucidated. Folate was originally thought to be reduced to THF upon entering the mucosal cell by the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH)-dependent enzyme dihydrofolate reductase (DHFR). The enzyme methylenetetrahydrofolate reductase (MTHFR) would then methylate THF, forming 5-methylTHF [1]. However, it has recently been suggested that metabolism is not required for transport [47]. Higher doses of folate may result in transport of the vitamin unchanged into the portal circulation, where folate may be metabolized to 5-methylTHF in the liver or may remain unchanged and appear in the peripheral circulation.

Typically, once folate enters the liver, it is stored in the liver, secreted into the bile, or enters the systemic circulation [46]. For storage, different forms of folates are converted into polyglutamates by the enzyme folylpolyglutamate synthetase (FPGS) **(Figure 2.2)** [1]. The addition of glutamate residues allows folate to be retained inside the cell, and polyglutamylated folates are better substrates than monoglutamates for intracellular folate-dependent enzymes. In contrast, the lysosomal enzyme γ-glutamyl hydrolase (GGH) catalyzes the hydrolysis of the polyglutamate chain, converting polyglutamate forms of folate in the cell into monoglutamate forms prior to the release of folate into the bile or blood [46, 48]. Folate can also be re-circulated by the enterohepatic cycle, where it is secreted into the bile, returned to the duodenum and jejunum, and is subsequently reabsorbed [46]. In addition, folate can leave the liver, enter the hepatic vein, and ultimately enter the systemic

circulation [46]. In the blood, folate exists in the monoglutamate form, consisting mainly of THF, 5-methylTHF, and 10-formylTHF, with most folate bound to proteins [1, 49]. Numerous studies have also detected the presence of unmetabolized folic acid in the systemic circulation [50-52].

Several different transport systems are involved in the cellular uptake of folate **(Figure 2.2)**. Passive diffusion is the first transport system; however, due to the hydrophilic and anion nature of folate, this is likely minimal, only occurring at pharmacological doses [46]. The second transport system involves the reduced folate carrier (RFC). RFC is an anionic exchanger, and appears to function optimally at physiological pH [46]. RFC has a greater affinity for 5-methylTHF and 5-formylTHF (K_m ~2-7 μM) than for folic acid (K_i ~150-200 μM) [46]. The third transport system involves the folate receptor (FR), often referred to as the folate binding protein (FBP). FR is a glycosylphosphatidylinositol anchored protein, and has several different isoforms [53]. The different isoforms of FRs differ significantly in their relative affinities for folate; however, they bind folic acid with a uniformly high affinity [53]. The final transport system involves the proton-coupled folate transporter (PCFT). PCFT was originally identified as a low-affinity heme transporter, while recent studies suggest that the primary role of this transporter is as a proton-dependent, high-affinity folic acid transporter optimally functioning at a low pH [46]. The expression of PCFT in the human duodenum is highly variable between individuals, and appears to be significantly higher in the duodenum compared with the ileum and colon [54]. PCFT has similar affinity (K_t ~ 0.5-0.8 μM at pH 5.5) for reduced folates and folic acid [55].

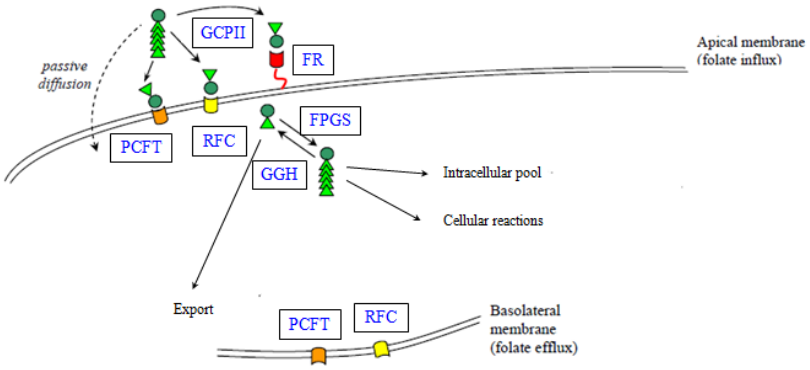

Figure 2.2 Simplified diagram of intestinal absorption and intracellular distribution of folate. Boxes outlined in black represent key enzymes involved in intraluminal folate hydrolysis (GCPII, glutamate carboxypeptidase II), intracellular folate uptake (FR, folate receptor; PCFT, proton-coupled folate transporter; RFC, reduced folate carrier), intracellular folate retention (FPGS, folylpolyglutamyl synthase) and hydrolysis and efflux (GGH, γ-glutamyl hydrolase). Each filled circle represents a pteridine ring conjugated to para-aminobenzoic acid. Each triangle represents a glutamate, which is linked via a peptide bond to form various chain lengths of polyglutamylated folate. Modified from [2].

2.1.4 Biochemical Role of Folate

The sole biological role of folate is to mediate the transfer of one-carbon moieties involved in nucleotide synthesis, the methionine cycle, and biological methylation reactions [2] **(Figure 2.3)**. In the methionine cycle, folate in the form of 5-methylTHF transfers a methyl group to homocysteine, forming methionine [1]. The formation of methionine ensures the provision of SAM, the primary methyl group donor for most biological methylation reactions, including that of DNA. The remethylation of homocysteine to methionine is catalyzed by the enzyme methionine synthase (MTR), which relies on cobalamin (vitamin B_{12}) as a cofactor [2]. The enzyme methionine synthase reductase (MTRR) is responsible for the reductive methylation of the cobalamin cofactor of MTR to its active state. Once 5-methylTHF has transferred a methyl group to homocysteine, THF is formed and is

subsequently converted to 5,10-methyleneTHF by serine hydroxymethyltransferase (SHMT). In the thymidylate synthesis pathway, 5,10-methyleneTHF is the one-carbon donor for the thymidylate synthase (TS) catalyzed nonreversible conversion of deoxyuridine-5-monophosphate (dUMP) to deoxythymidine-5-monophosphate (dTMP) [1]. The synthesis of dTMP results in the oxidation of 5,10-methyleneTHF to the inactive DHF. DHF is then converted into THF by DHFR. THF and 5,10-methyleneTHF can also enter a pathway involved in the synthesis of purines (adenine and guanine) by the addition of a formyl group, forming 10-formylTHF.

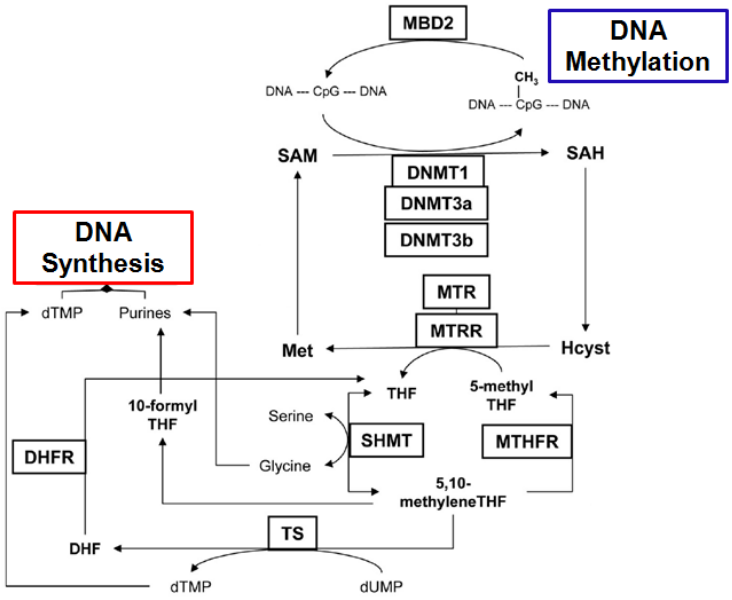

Figure 2.3 Simplified scheme of the biochemical roles of folate. Folate mediates the transfer of one-carbon moieties necessary for DNA synthesis, the methionine cycle, and biological methylation reactions. Boxes outlined in black represent key enzymes involved in the methionine cycle (MTR, methionine synthase; MTRR, methionine synthase reductase; MTHFR, 5,10-methylenetetrahydrofolate reductase), maintenance of intracellular pool (DHFR, dihydrofolate reductase; SHMT, serine hydroxylmethylenetransferase), nucleotide biosynthesis (TS, thymidylate synthase), DNA methylation (DNMT1, 3a, 3b, DNA methyltransferases), and DNA demethylation (MBD2, DNA demethylase). CH_3, methyl group; CpG, cytosine-guanine dinucleotide sequence; dTMP, deoxythymidine-5-monophosphate (thymidylate); dUMP, deoxyuridine-5-monophosphate; Hcyst, homocysteine; Met, methionine; THF; tetrahydrofolate, SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine. Modified from [2].

2.1.5 Folate and Health

Folate plays a critical role in human health and disease, and in general, has been considered safe and an ideal functional food component for disease prevention [56]. Epidemiologic studies suggest folate deficiency is associated with a number of disorders including anemia, NTDs and other congenital defects, atherosclerosis, adverse pregnancy outcomes, neuropsychiatric disorders, osteoporosis, and certain cancers [56-58].

2.1.5.1 Dietary Reference Intakes of Folate: RDA and UL

Dietary Reference Intakes (DRIs) consist of several nutrient-based reference values, including the Estimated Average Requirement (EAR), Recommended Dietary Allowance (RDA), and the Tolerable Upper Intake Level (UL) [41]. The EAR is defined as the median usual intake value that is estimated to meet the requirement of half the healthy individuals in a life stage and gender group, and is used to determine the RDA. The average daily intake level that is sufficient to meet the nutrient requirement of most (97-98%) healthy individuals is referred to as the RDA. **Table 2.2** lists the EAR and RDA values of folate for different age groups and during pregnancy and lactation.

Table 2.2 EAR and RDA values of folate (μg/day of DFEs) for different age groups and during pregnancy and lactation

Age and Gender Group	EAR (μg/day of DFEs)	RDA (μg/day of DFEs)
1-3 years	120	150
4-8 years	160	200
9-13 years	250	300
14-18 years	330	400
19-30 years	320	400
31-50 years	320	400
≥51 years	320	400
Pregnancy	520	600
Lactation	450	500

*EAR=Estimated Average Requirements; RDA=Recommended Dietary Allowance; DFEs=dietary folate equivalents

The EAR for children was extrapolated from adult values for the different age categories (1-3, 4-8, 9-13, and 14-18 years) [41]. The RDA for children and adolescents was set using the respective EAR for each age category plus twice the coefficient of variation. For adult males and nonpregnant females (≥19 years), RBC folate concentration was the primary indicator that was used to evaluate adequacy of folate intake to maintain status and to determine the EAR and RDA. The EAR for adults was mainly based on data from controlled metabolic studies, and the RDA was then set using this value.

The UL is considered the maximum daily nutrient intake that is not likely to result in adverse health effects in the majority of the population [59]. The UL for adult (≥19 years) men and women, including pregnant and lactating women, was set by the Institute of Medicine at 1000 μg/day of folic acid either from fortified foods or as a supplement, excluding folates found naturally in foods [41]. This particular value was chosen to ensure that the diagnosis of macrocytic anemia due to vitamin B_{12} deficiency would not be masked by high levels of folic acid [60]. The Institute of Medicine used the adult UL value to

extrapolate age-specific ULs for children [61]. For children aged 1-3, 4-8, 9-13, and 14-18 years, the ULs are 300, 400, 600, and 800 μg/day, respectively [41].

2.1.5.2 Folate and Pregnancy

2.1.5.2.1 RDA during Pregnancy

Pregnancy is accompanied with a drastic acceleration in one-carbon transfer reactions, including those required for nucleotide synthesis, in order to ensure rapid cell division necessary for the increase in red cell mass, enlargement of the uterus, and growth of both the placenta and fetus [62]. Due to the essential role folate plays in DNA synthesis, the requirement for folate increases substantially during pregnancy [62]. The increased demand for folate, however, may also place pregnant women at greater risk of developing folate deficiency. Thus, it is important for pregnant women to ensure adequate folate intake throughout pregnancy, as folate deficiency is associated with numerous adverse pregnancy outcomes [63]. For pregnant women, the RDA for folate is 50% higher than the value set for adult men and nonpregnant women, and was calculated based on data from a controlled metabolic study and a series of population-based studies in which dietary folate intake was reported [64]. The results from these studies consistently demonstrated that 600 μg/day DFEs was an adequate amount to maintain normal folate status and to sustain fetal tissue growth. Therefore, the RDA for pregnant women was set at 600 μg/day DFEs [41].

2.1.5.2.2 Folate Deficiency during Pregnancy and Neural Tube Defects

Folate deficiency during pregnancy has been associated with a variety of pregnancy complications, including placental abruption, preeclampsia, spontaneous abortion and stillbirth, low infant birth weight, and NTDs and other congenital defects in the offspring [63]. NTDs, the most common major congenital malformations of the central nervous system, are the result of inadequate closure of the developing neural tube in either the cranial region (anencephaly) or more caudally along the spine (spina bifida) [65].

Over 30 years ago, Smithells *et al*. observed a relationship between sub-optimal folate status and NTDs, as women with infants affected by NTDs were observed to have diets and postpartum blood levels low in several micronutrients, especially folate [66]. Following these observations, a substantial amount of evidence from observational studies and clinical trials in the 1980s and 1990s demonstrated that maternal folic acid supplementation was associated with a reduced risk of the occurrence and recurrence of NTDs [67-70].

2.1.5.2.3 Folate Recommendations and Guidelines

The Institute of Medicine published guidelines which currently advise women of childbearing age to consume 400 μg of folic acid daily from a combination of supplements and fortified foods, in addition to naturally occurring folates found in a well-balanced diet [41]. In Canada, Health Canada currently recommends that all women capable of becoming pregnant consume a multivitamin containing 400 μg of folic acid daily, in addition to dietary

folates (both naturally occurring folates and synthetic folic acid) obtained from a well-balanced diet [71].

Recently, the SOGC and The Motherisk Program have published guidelines for women of childbearing age, consisting of three different recommendations that vary according to age, ethnicity, compliance, and genetic congenital anomaly risk status [12]. Firstly, women with no personal health risks, a planned pregnancy, and good compliance, are advised to consume a daily multivitamin containing between 400 μg and 1 mg of folic acid in addition to a diet rich in folate, for at least 2-3 months before conception and throughout pregnancy and the postpartum period. Secondly, women with health risks such as epilepsy, insulin dependent diabetes, obesity, family history of NTDs, or those belonging to a high-risk ethnic group such as Celtic and Sikh, are advised to increase their dietary intake of foods rich in folate and are recommended to consume a daily multivitamin containing 5 mg folic acid, beginning 3 months before conception and continuing until 10-12 weeks post conception. For the remainder of pregnancy and the postpartum period, this specific group of women are advised to consume a daily multivitamin containing between 400 μg and 1 mg of folic acid. Lastly, women with a history of poor compliance with medications as well as other lifestyle issues such as no consistent birth control and possible teratogenic substance use are advised to consume a daily multivitamin containing 5 mg of folic acid.

2.1.5.3 Folate and Lactation

Breastfeeding is considered the optimal method of feeding infants, and Health Canada currently encourages exclusive breastfeeding for the first 6 months of life [72]. In human milk, the main form of folate is 5-methylTHF [72]. The concentration of folate

appears to vary considerably (ranging from 50-320 nmol/L); however, it depends on several factors. For example, the concentration of folate is typically lower in foremilk than in hindmilk, and lower in the morning compared to later on in the day. It has also been suggested that the concentration of folate in milk increases with the progression of lactation; however, data appears to be inconsistent, most likely due to the different sample collection procedures and assay methodologies.

It is important that human milk contains adequate levels of all the essential nutrients; thus, maternal nutritional requirements for these nutrients, including folate, are high during lactation. The Estimated Average Requirement (EAR) for lactating women is estimated as the folate intake necessary to replace the folate secreted daily in human milk in addition to the amount required to maintain maternal folate status. The average volume of milk produced (0.78L) was multiplied by the folate concentration of breast milk (85 μg/L), which was then multiplied by a bioavailability correction factor of 2. This amount is equal to 133 μg and was added to the EAR for nonpregnant, nonlactating women to determine the RDA. The RDA for lactating women was therefore set at 500 μg/day DFEs [41].

Folate concentrations in human milk appear to be tightly regulated, and with the exception of severe folate deficiency in the mother, folate concentrations are minimally influenced by maternal folate status [72]. In a recent 16-week randomized, placebo-controlled intervention, healthy lactating women were assigned to receive [6S]-5-methylTHF (416 μg/day) or a placebo, or were assigned to receive folic acid (400 μg/day) within 1 week after the birth of their infants [73]. Following 16 weeks of supplementation, mean concentrations of plasma folate in women that consumed folic acid or [6S]-5-methylTHF (97.0 nmol/L and 104 nmol/L, respectively) were significantly greater than those in the

placebo group (47.6 nmol/L), while milk folate concentrations were similar between the folic acid- and [6S]-5-methylTHF-supplemented groups and did not differ from those women in the placebo group. Interestingly, unmetabolized folic acid was detected in 96% of milk samples, and represented approximately 8% of total milk folate concentrations.

2.1.5.4 Mandatory Folic Acid Fortification

On the basis of available evidence regarding the protective role of folic acid supplementation against NTDs, the United States Public Health Service in 1992 issued a recommendation that all women of childbearing age capable of becoming pregnant consume 400 µg/day of folic acid [74]. However, poor compliance with these recommendations was evident. For example, in a survey conducted in 1998 to assess folic acid knowledge and practices among women of childbearing age in the United States, only 7% of women were aware of the importance of periconceptional folic acid supplementation in reducing the risk for NTDs [75]. In addition, in the United States, as many as 50% of pregnancies are thought to be unplanned [76]. In order to increase folate intake among women of childbearing age, fortification of the food supply with folic acid was considered the most effective approach; therefore, in 1998, mandatory fortification of certain food products with folic acid was implemented in both the United States and Canada [74]. In the United States, all enriched cereal grain products, such as flour, rice, breads, rolls and buns, pasta, were to be fortified with folic acid at 140 µg of folic acid per 100 g of flour, while in Canada, white flour and cornmeal were to be fortified at 150 µg/100 g and pasta was to be fortified at 200-270 µg/100 [77, 78]. Recently, in Canada, there have been calls to further increase the level of the folic acid in fortified foods [12].

Several other countries have implemented mandatory fortification programs in addition to the United States and Canada, including Chile and Costa Rica, while many other countries have not yet followed [79]. In Australia and New Zealand, voluntary fortification of certain food products has been introduced. In Europe, fortification policies vary considerably. Germany, for example, allows fortification of flour and breakfast cereals, while other countries such as the United Kingdom have decided to delay mandatory folic acid fortification to further evaluate concerns and potential risks in specific groups of the population [80].

2.1.5.5 Postfortification Era

2.1.5.5.1 Blood Folate Status

Numerous studies have examined the effect of mandatory folic acid fortification on biochemical measurements of folate status (**Table 2.3**). In the United States, a significant increase in mean plasma folate concentrations (11 nmol/L to 23 nmol/L) and a significant decrease in mean plasma homocysteine concentrations (10.1 μmol/L to 9.4 μmol/L) were observed following mandatory folic acid fortification in blood samples examined from middle-aged and older men and women in the Framington Offspring Study cohort [81].

Data from the National Health and Nutrition Examination Survey (NHANES) have also shown significant improvements in the folate status of the United States population (≥ 4 years) following mandatory folic acid fortification [9]. Following these large increases, a slight downward trend in both RBC and serum folate concentrations have been observed [9]. The most recent NHANES data (2005-2006) suggest there has been essentially no change in

RBC and serum folate concentrations from the previous two year period in persons ≥4 years of age [10]. Pfeiffer *et al*. have also examined homocysteine data from the prefortification and postfortification NHANES survey periods, and observed decreases in adolescent, adult and older men and women from before to after fortification [82]. Several periods examined in the postfortification era, however, suggest that concentrations of homocysteine have remained relatively constant.

In women of childbearing age (15-45 years) in the United States, median serum folate levels were shown to significantly increase (4.8 ng/mL to 13.0 ng/mL) from the prefortification survey period to the initial postfortification (1999-2000) survey period [10]. This large increase was followed by a slight downward trend in median serum folate levels. The most recent (2005-2006) NHANES median serum folate value for women of childbearing age is 11.4 ng/mL, a value remaining well above that of the prefortification period [10].

A significant increase in blood folate levels and decrease in homocysteine concentrations following the implementation of folic acid fortification have also been observed in Canada and Chile. In a retrospective cross-sectional study using a large Ontario laboratory database, the geometric mean serum folate level in adult men and women was shown to significantly increase (18.5 nmol/L to 27.1 nmol/L) from 1997-1998 and 1999-2000, and similarly, a significant increase (680.3 nmol/L to 851.6 nmol/L) in RBC folate was observed [83]. Ray *et al*. also examined the effect of the fortification program among Ontario women of reproductive age (18-42 years), and found that the geometric mean RBC folate concentration significantly increased (527 nmol/L to 741 nmol/L) from prefortification to postfortification [84]. In a population based study in Newfoundland, a significant increase in

both serum and RBC folate concentrations, and a corresponding decrease in mean plasma homocysteine, were observed in women (19-44 years) and seniors (≥65 years) following the implementation of mandatory folic acid fortification [85]. In Chile, the mean serum folate concentration significantly increased (9.7 nmol/L to 37.2 nmol/L) and the mean RBC folate concentration significantly increased (290 nmol/L to 707 nmol/L) from prefortification to postfortification in women of childbearing age [86].

Table 2.3 Effect of mandatory folic acid fortification on biochemical measurements of blood folate status in various age and gender groups

Study	Country	Age/Gender Group	Prefortification vs. Postfortification	Folate Concentration Prefortification vs. Postfortification		Homocysteine
Jacques *et al*. 1999 [81]	United States	Middle-aged and older men & women	1991-1994 vs. 1995-1998	Plasma (mean) 11 nmol/L vs. 23 nmol/L (p<0.001)		Plasma tHcy (mean) 10.1 vs. 9.4 μmol/L (p<0.001)
Pfeiffer *et al*. 2007 [9]	United States	NHANES Males and females 4-11 years	1988-1994 vs. 1999-2000, 2001-2002, 2003-2004,	Serum (median)¶ 8.8 ng/mL vs. 19.3 ng/mL (1999-2000) (p<0.001) 17.0 ng/mL (2001-2002) (NS) 15.6 ng/mL (2003-2004) (p<0.002)	RBC (median)¶ 200 ng/mL vs. 287 ng/mL (1999-2000) (p<0.001) 274 ng/mL (2001-2002) (NS) 256 ng/mL (2003-2004) (p=0.002)	Not measured
		NHANES Males and females 12-19 years		Serum (median)¶ 5.1 ng/mL vs. 13.3 ng/mL (1999-2000) (p<0.001) 12.6 ng/mL (2001-2002) (NS) 11.0 ng/mL (2003-2004) (p=0.003)	RBC (median)¶ 150 ng/mL vs. 244 ng/mL (1999-2000) (p<0.001) 237 ng/mL (2001-2002) (NS) 224 ng/mL (2003-2004) (p=0.003)	
		NHANES Males and females 20-59 years		Serum (median)¶ 4.8 ng/mL vs. 12.5 ng/mL (1999-2000) (p<0.001) 11.8 ng/mL (2001-2002) (NS) 11.0 ng/mL (2003-2004) (p<0.001)	RBC (median)¶ 164 ng/mL vs. 269 ng/mL (1999-2000) (p<0.001) 271 ng/mL (2001-2002) (NS) 248 ng/mL (2003-2004) (p<0.001)	
		NHANES Males and females ≥60 years		Serum (median)¶ 6.9 ng/mL vs. 17.4 ng/mL (1999-2000) (p<0.001) 16.6 ng/mL (2001-2002) (NS) 15.6 ng/mL (2003-2004) (NS)	RBC (median)¶ 211 ng/mL vs. 344 ng/mL (1999-2000) (p<0.001) 335 ng/mL (2001-2002) (NS) 321 ng/mL (2003-2004) (NS)	
McDowell *et al*. 2009 [10]	United States	Women of reproductive age 15-45 years	1988-1994 vs. 2005-2006	Serum folate (median)¶ 4.8 ng/mL vs. 11.4 ng/mL	RBC folate (median)¶ 160 ng/mL vs. 257 ng/mL	Not measured
Ray *et al*. 2002 [83]	Canada	Adult men & women	1997-1998 vs. 1998-1999	Serum folate (geometric mean) 18.5 nmol/L vs.	RBC folate (geometric mean) 680.3 nmol/L vs.	Not measured

		57.4 years (mean)		27.1 nmol/L (p<0.001)	851.6 nmol/L (p<0.001)	
Ray *et al*. 2002 [84]	Canada	Women of reproductive age 18-42 years	1996-1997 vs. 1998-2000	RBC folate (geometric mean) 527 nmol/L vs. 741 nmol/L		Not measured
Liu *et al*. 2004 [85]	Canada	Women of reproductive age 19-44 years	1997-1998 vs. 2000-2001	Serum folate (geometric mean) 13.5 nmol/L vs. 18.1 nmol/L (p<0.001)	RBC folate (geometric mean) 625 nmol/L vs. 818 nmol/L (p<0.001)	Plasma tHcy (geometric mean) 10.2 µmol/L vs. 9.2 µmol/L (p=0.001)
		Seniors ≥65 years		Serum folate (geometric mean) 14.8 nmol/L vs. 23.0 nmol/L (p<0.001)	RBC folate (geometric mean) 745 nmol/L vs. 916 nmol/L (p<0.001)	Plasma tHcy (geometric mean) 13.6 µmol/L vs. 12.3 µmol/L (p=0.001)
Hertrampf *et al*. 2003 [86]	Chile	Women of reproductive age 29.6 years (mean)	1999 vs. 2000	Serum folate (mean) 9.7 nmol/L vs. 37.2 nmol/L (p<0.0001)	RBC folate (mean) 290 nmol/L vs. 707 nmol/L (p<0.0001)	Not measured

*tHcy=total homocysteine; NHANES= National Health and Nutrition Examination Survey; RBC=red blood cell; NS=not significant

¶To convert values for folate to nanomoles per litre, multiply by 2.266

2.1.5.5.2 Dietary Folate Intake and Folic Acid Content in Fortified Foods

Initially, it was estimated that the average increase of folic acid intake in the general population attributable to the intervention of mandatory folic acid fortification would be 100 µg/day [74]. However, using linear regression analysis of data from published studies to quantify the increase in folic acid intake from fortification, Quinlivan and Gregory estimated that intakes of folic acid from fortified foods were more than twice the amount originally predicted (>200 µg/day) [87]. Furthermore, a study using data on food and nutrient intake from individuals who participated in the 5th and 6th examinations of the Framington Offspring Cohort Study found that folic acid intake increased by a mean of 190 µg/day and total folate intake increased by a mean of 323 µg DFEs/day following fortification [74].

Overage of folic acid added in the process of fortification may contribute to the greater than expected increase in daily folic acid intake. Recently, a Canadian study examined a variety commonly purchased foods that are fortified with folic acid and found that the actual folate content in many of these products was greater than the values reported in Canada's main reference nutrient database and the values on food labels [78]. In Canada, a regulated upper limit to the amount of folic acid that can be added to foods has not been set; therefore, manufacturers may frequently fortify foods at higher levels than those values mandated [88].

2.1.5.5.3 Supplemental Use of Folic Acid

It has been estimated that approximately 30-40% of the North American population consumes supplements [11]. In the United States, NHANES data suggest that the use of

supplements containing folic acid was greater immediately following mandatory folic acid fortification (1999-2000) than the prefortification period (1988-1994) [11]. Among NHANES 2003-2006 participants, Bailey *et al*. have recently reported that the use of dietary supplements was 53.4%, while the use of supplements containing folic acid was 34.5% [89].

The use of supplements appears to be particularly high in children. Using data from the 2004 Canadian Community Health Survey, Shakur *et al*. recently found that Canadian children (4-8 years) were the age group most likely to consume supplements containing folic acid [88]. In another study, 30% of preschoolers (3-5 years) in Ontario were reported to consume supplements containing folic acid [90]. Using NHANES III (prefortification) data, Balluz *et al*. found that 42-51% of children (1-5 years) in the United States consumed a supplement with or without folic acid [91]. In a study using more recent NHANES data (2003-2006), Yeung *et al*. found that in the United States, 26% of children (1-18 years) consumed folic acid from supplements, with the highest proportion being children 4-8 years [61].

Among females of reproductive age in Canada, 15%, 22.9%, and 29.2% of females aged 14-18, 19-30, and 31-50 years, respectively, were reported to consume supplements containing folic acid [88]. Furthermore, 28.5% of women >70 years of age were reported to consume folic acid supplements. Liu *et al*. found that the proportion of women in Newfoundland taking a vitamin supplement containing folic acid increased substantially (from 17% to 28%) between prefortification (1997-1998) and postfortification (2000-2001) [85]. In the United States, NHANES 2001-2002 data suggested that non-Hispanic white women obtained approximately 48% of their folic acid intake from supplements, while non-Hispanic black and Hispanic women obtained approximately 26% [92].

2.1.5.5.4 Neural Tube Defects

Mandatory folic acid fortification has been deemed a public health triumph, in the sense that studies suggest a 15-50% reduction in the incidence and prevalence of NTDs in the United States and Canada [5-8]. In the United States, several studies have reported percent decreases in the overall birth prevalence rate of NTDs following the initiation of folic acid fortification in 1998 [5, 7]. In Canada, a 46% reduction in NTDs from prefortification to postfortification was observed; however, the rates have been shown to vary between provinces and the risk reduction was shown to be greatest in those provinces in which the NTD rates were highest before the initiation of fortification [93]. In Chile, folic acid fortification of wheat flour for bread (220 μg folic acid/100 g) was associated with a 43% reduction in NTDs [65].

2.1.5.5.5 Potential Concerns of High Folate Status

Due to the substantial increase in folate status throughout the North American population following fortification, it has been suggested that certain segments of the population may be exceeding the UL [94]. Using NHANES data from 2003-2006, 0.4-5.2% of older adults (≥ 60 years) and close to 3% of adults (≥19 years) were shown to have intakes exceeding the UL [94, 95]. However, Yang *et al*. recently demonstrated that only those adults consuming supplements containing folic acid appeared to exceed the UL [94]. It is important to note that both of these studies exclude young children, an age group that was shown to have the highest serum folate concentrations in the United States in recent NHANES data [9, 96]. The UL for children aged 1-3 years and 4-8 years is set at 300 μg/day

and 400 µg/day, respectively [41]. Thus, many children in North America may be exceeding this level. Interestingly, using data from the 2004 Canadian Community Health Survey, Shakur *et al*. recently found that the prevalence of folate inadequacy in children (< 14 years) was very low, and in addition, children (4-8 years) were the age group most likely to consume supplements containing folic acid [88]. The authors concluded that regulatory guidance allowing for the inclusion of folic acid in supplements designed for children should be reassessed [88].

Emerging evidence suggests that a high folate status may be associated with several adverse outcomes (**Table 2.4**). It has been suggested that high levels of folic acid may delay the diagnosis of vitamin B_{12} deficiency, allowing vitamin B_{12} deficiency-induced neurological disorders to progress [37]. Although this is likely rare, certain segments of the population, such as the elderly, are of particular concern, as decreases in vitamin absorption and extraction of vitamin B_{12} from protein have been shown to occur with the aging process [97]. In senior Americans (≥ 60 years) with normal vitamin B_{12} status, high serum folate (>59 nmol/L) was associated with protection from cognitive impairment, while among seniors with low vitamin B_{12} status (<148 pmol/L), high serum folate was directly related to anemia and cognitive impairment [97]. A recent single-centre, randomized, double-blind controlled trial in seniors (>70 years of age) demonstrated that supplementation with folic acid (800 µg/day) in addition to vitamins B_{12} and B_6 slowed the accelerated rate of brain atrophy observed in mild cognitive impairment [98].

In postmenopausal women (50-75 years of age), Troen *et al*. observed an inverse U-shaped relationship between dietary folate or supplemental folic acid and natural killer cell (NK) cytotoxicity, an index of immune function that is related to cancer cell surveillance

[50]. Postmenopausal women with a low dietary intake of folate (<233 μg/day) and that consumed up to 400 μg/day of supplemental folic acid demonstrated higher mean NK cytotoxicity. In contrast, in women with greater dietary folate intakes (>233 μg/day), supplemental folic acid showed no additional benefits, and women with higher dietary intakes of folate who consumed >400 μg/day from folic acid demonstrated significantly lower NK cytotoxicity compared to those consuming a diet low in folate and no supplements. Furthermore, a highly significant inverse linear association between unmetabolized folic acid concentrations in plasma and natural killer cell cytotoxicity was observed, while no association was observed between total plasma folate and NK cytotoxicity.

Recent studies have also suggested that high folate status may have no additional benefit or may even increase the risk of certain cancers [99-104]. In the Aspirin/Folate Polyp Prevention Study, folic acid supplementation (1 mg/day) was associated with a 67% increased risk of advanced lesions in the colon with a high malignant potential, along with a two-fold increased risk of having ≥3 colorectal adenomas [103]. Furthermore, the risk of prostate cancer was significantly increased in the folic-acid supplemented group [104]. In a recent combined analysis and extended follow-up of participants from 2 randomized, double-blind, placebo-controlled clinical trials, patients with ischemic heart disease were treated with folic acid (800 μg/day) plus vitamin B_{12} (400 μg/day) and vitamin B_6 (0.04 μg/day), folic acid (800 μg/day) plus vitamin B_{12} (400 μg/day), vitamin B_6 alone (0.04 μg/day), or placebo [105]. Interestingly, treatment with folic acid combined with vitamin B_{12} was associated with increased cancer outcomes and all-cause mortality in patients with ischemic heart disease in Norway, a country that does not fortify foods with folic acid.

While data from animal studies generally support a causal relationship between folate deficiency and colorectal cancer (CRC) risk and an inhibitory effect of modest levels of folate supplementation on colorectal carcinogenesis, it has recently been suggested that folate may possess dual modulatory effects on colorectal carcinogenesis depending on the timing and dose of folate intervention [2].

Prior to folic acid fortification, both the United States and Canada were shown to experience a decreasing downward trend in CRC incidence [106]. However, absolute rates of CRC began to rise in the United States and in Canada, around the time of the initiation of mandatory folic acid fortification in these countries. Thus, it has been suggested that folic acid fortification may have been wholly or partly responsible for these observed increases in CRC rates. Interestingly, a recent study reported substantial increases in the use of folic acid-containing supplements in patients following the diagnosis of CRC [107].

A high folate status may also be associated with a reduction in the effectiveness of anti-folate drugs used against malaria, rheumatoid arthritis, psoriasis, and cancer [108]. In addition, a high maternal folate status during pregnancy may be associated with several adverse outcomes in the offspring [15, 16, 109]. ***Chapter 2.2.4*** will discuss these adverse outcomes in greater detail.

Table 2.4 Examples of recent human studies suggesting potential adverse effects associated with high folate status

Potential Adverse Effect	Reference	Outcome in Study
Masking of vitamin B_{12} deficiency	Morris *et al*. 2007 [97]	High serum folate (>59 nmol/L) and low vitamin B_{12} (<148 pmol/L) associated with anemia and cognitive impairment in seniors High serum folate (>59 nmol/L) and normal vitamin B_{12} status associated with protection against cognitive impairment
Decreased natural killer cell cytotoxicity	Troen *et al*. 2006 [50]	Postmenopausal women with low dietary folate intake (<233 μg/day) and used folic acid supplements had greater NK cytotoxicity, while those with folate-rich diet and used folic acid supplements (>400 μg/day) had reduced NK cytotoxicity Inverse relationship between unmetabolized folic acid in plasma and NK cytotoxicity
Cancer (mainly of colon, breast, and prostate)	Cole *et al*. 2007 [103]	Folic acid supplementation (1 mg/day) associated with increased risk of advanced lesions in colon, having ≥3 adenomas in colon, and noncolorectal cancers
	Stolzenberg-Solomon *et al*. 2006 [102]	Increased risk of breast cancer in postmenopausal women who consumed folic acid supplements (>400 μg/day) and postmenopausal women in highest quintile of total folate intake (>853 μg/day)
Reduced efficacy of or increased resistance to antifolate drugs	Khanna *et al*. 2005 [110]	Patients taking 1-2 mg folic acid/day had poorer clinical response to methotrexate
Asthma in offspring[¶]	Whitrow *et al*. 2009 [17]	Supplemental form of folic acid taken (300 μg/day=median) in late pregnancy associated with increased risk of childhood asthma at 3.5 years and with persistent asthma
Increased adiposity and insulin resistance in offspring[¶]	Yajnik *et al*. [15]	Children (6 years) whose mothers during pregnancy had high RBC folate concentrations (28 weeks of pregnancy) had a greater total fat mass and insulin resistance Children (6 years) whose mothers during pregnancy had low vitamin B_{12} with high RBC folate concentrations (28 weeks of pregnancy) were the most insulin resistant

* NK=natural killer cell; RBC=red blood cell

[¶]Adverse effect in offspring as a result of a high maternal folate status

2.2 Influence of Maternal Nutrition on the Developing Offspring

2.2.1 Developmental Plasticity

Organisms are considered 'plastic' in the sense that they are able to adapt to their environment. 'Developmental plasticity' refers to the manner in which one genotype is capable of giving rise to a range of phenotypes in response to environmental conditions during development [111]. In response to environmental cues, the organism will often choose a trajectory of development that has an adaptive advantage [112]. One example of this is the microcrustacean *Dapnia*, where mothers exposed to chemicals from predators will produce offspring with a defensive helmet-like structure [14]. In humans, a mother that is poorly nourished will signal to the developing embryo and fetus that the environment in postnatal life is likely to be similar and therefore, the developing organism can respond to these signals by making adaptations, such as reducing body size and altering metabolism [111]. During early life development, there is a specific time in which developmental plasticity occurs, and it has been suggested that this period of developmental plasticity may extend from the time of conception, or possibly preconception, into the early postnatal period [112].

2.2.2 Early Life Origins of Disease

The 'developmental origins of adult disease' hypothesis states that adverse environmental influences during the *in utero* and early postnatal periods can lead to permanent changes in physiology and metabolism resulting in an increased risk of disease in adulthood [113]. Initially termed the 'fetal origins hypothesis', this notion originated over

two decades ago, following observations by Barker and colleagues that certain regions of England and Wales with the highest rates of infant deaths between 1921 and 1925 also had, decades later, the highest rates of death from coronary heart disease [114]. The hypothesis was recently renamed to the 'developmental origins hypothesis', upon the realization that developmental plasticity could extend into the postnatal period [112].

Following their initial observations, Barker and colleagues observed an inverse relationship between birth weight and adult coronary heart disease morbidity in a large sample of men from Hertfordshire born between 1911 and 1930, further supporting the notion that intrauterine deprivation is an important mediator in cardiovascular disease [115]. These results were replicated in women born during this time period, as well as several other studies in Europe and the United States [116-118]. Soon thereafter, it was suggested that low birth weight may increase the risk of numerous diseases, such as type 2 diabetes mellitus, hypertension, obesity, and osteoporosis [14]. In contrast, the risk of certain cancers has been suggested to be associated with high birth weight [14].

2.2.3 Maternal Nutrition and the Induction of Altered Phenotypes in the Offspring

The honeybee (*Apis mellifera*) model is an excellent example of the impact of nutrition early in life on the induction of alternative phenotypes in the offspring [119]. Female bees have identical genomic sequences and therefore are essentially genetic clones. Interestingly, however, queen and worker bees differ substantially in their morphology, capacity to reproduce, behaviour, and longevity, and these differences are the result of female larvae fed different diets [119]. Although all larvae are fed following hatching, those larvae fed royal jelly, a mixture of proteins, amino acids, vitamins, lipids, and other nutrients

that has not been completely defined at present, will become queen bees [119]. In contrast, larvae fed less-sophisticated food will develop as worker bees [119].

Numerous early life environmental exposures may induce phenotypic changes and ultimately increase the risk of disease in the offspring; however, for the purpose of this literature review, the main focus will be maternal nutrition. One of the most well-known examples of the role that maternal nutrition plays in the offspring's risk of disease later in life stems from the Dutch Hunger Winter, a severe famine that occurred in the western part of The Netherlands as a result of the Nazi blockade between 1944 and 1945 [14]. During the famine, many women continued to become pregnant despite a drastic reduction in daily calorie intake from 1800 kcal to between 400 and 800 kcal [120]. Numerous studies have demonstrated an association between prenatal exposure to this famine and an increased risk of obesity, mood disorders, impaired glucose and lipid homeostasis, and reduced renal function [121]. Furthermore, the effect of famine on the offspring has been shown to differ depending on gestational age at the time of exposure [120]. In addition to observations from the Dutch Hunger Winter, an interventional study demonstrated that offspring of women from Scotland who consumed 0.45 kg meat and avoided carbohydrates during pregnancy had increased blood pressure and blood cortisol concentrations in adulthood [122, 123].

Animal models have also provided evidence that alterations in maternal nutrition, such as protein restriction or global dietary restriction, throughout specific periods of pregnancy and/or lactation can induce phenotypic changes in the offspring which may ultimately lead to increased disease risk [14]. Reducing maternal nutrient intake during pregnancy has been shown to result in intrauterine growth retardation, impaired cholesterol homeostasis, and altered development of the fetal hypothalamic-pituitary-adrenal axis

producing a reduction in pituitary and adrenal responsiveness to endogenous stimuli [124-126]. Maternal dietary protein restriction throughout pregnancy has been shown to lead to a variety of adverse outcomes including hypertension, impaired lipid and glucose homeostasis, and vascular dysfunction [127-130].

2.2.4 Influence of Maternal Folate Status on Health Outcomes in the Offspring

The relationship between a mother's folate status during pregnancy and/or during lactation and health outcomes in the child is becoming a topic of growing investigation. Numerous studies suggest that maternal folate status can influence the development and health outcomes of her progeny. For example, maternal folate deficiency during pregnancy has been associated with a wide variety of adverse pregnancy outcomes including placental abruption, preeclampsia, spontaneous abortion and stillbirth, and NTDs and other congenital defects in the offspring [63]. However, the effects of high maternal folate status on health outcomes in the offspring are less clear.

To date, most studies have focused on the protection of high folate status against major developmental abnormalities including NTDs, as well as the role of folate in the treatment of anemia. However, emerging evidence suggests that maternal folate supplementation may also have other favourable health outcomes in the offspring. In rodents, a maternal diet low in protein has been shown to elevate blood pressure in the offspring, while the addition of folic acid to the maternal diet prevented this elevation and several adverse vascular changes [130]. Sie *et al*. recently found that maternal folic acid supplementation throughout pregnancy and lactation was associated with morphologic changes of the mammary glands in offspring that may be associated with a reduction in

mammary tumor risk in the offspring [131]. Furthermore, Sie *et al*. have recently reported that maternal folic acid supplementation significantly reduces CRC risk in the offspring in animal model [132].

In humans, a population-based case-control study in children from Western Australia found that folate supplementation in pregnancy reduced the risk of common acute lymphoblastic leukaemia (ALL) [133]. In Canada, a recent population-based cohort study of all incident cases of childhood malignancy in Ontario between 1985 and 2006 found no significant change in the risk of ALL, brain cancers, or embryonal cancers between the prefortification to postfortification time periods; however, an approximately 30% reduction in risk of Wilms' tumor was observed following the initiation of mandatory folic acid fortification [134]. In addition, another Canadian study found that mandatory folic acid fortification was associated with a 60% reduction in neuroblastoma, but did not change the rate of infant ALL or hepatoblastoma [135].

However, recent reports suggest that a high maternal folate status may also be associated with various undesirable health outcomes in the offspring. In rats, male offspring from mothers that consumed a diet low in protein supplemented with folic acid during pregnancy demonstrated a decrease in growth rate after 7 months, an increase in blood glucose and corticosterone concentrations, and 40% lower brain concentrations of docosahexaenoic acid compared to offspring from mothers on the control diet [136, 137]. In addition, Ly *et al*. found that in a dimethylbenzanthracene (DMBA) rodent model, intrauterine and early postnatal exposure to supplemental levels of folic acid increased the risk of breast cancer development in the offspring [138].

In humans, an increased risk of neuroblastoma with maternal folate supplementation has been reported [139]. Furthermore, in the Pune Maternal Nutrition Study in India, maternal macronutrient intakes appeared to be unrelated to adiposity and insulin resistance in the offspring; however, maternal serum folate levels were positively associated with adiposity and insulin resistance in offspring at 6 years of age. Offspring of mothers deficient in vitamin B_{12}, but folate replete, demonstrated the greatest degree of insulin resistance [15]. Additionally, the recent Norwegian Mother and Child Cohort Study demonstrated that supplementation with folic acid during the first trimester of pregnancy was associated with increased risk of wheeze and respiratory tract infections in offspring up to 18 months of age [16].

2.2.4.1 Potential Mechanism: Epigenetics and DNA Methylation

While environmental stimuli, including nutritional factors, during prenatal and early postnatal mammalian development have been shown to induce phenotypic changes and potentially increase the risk of developing chronic disease later in life, the underlying biological mechanisms at present are less clear [113]. One plausible mechanism may be through epigenetic mechanisms, including DNA methylation, since persistent changes in the offspring phenotype must ultimately involve stable changes in gene expression [140]. A recent study using monozygotic and dizygotic twin pairs demonstrated the highly sensitive state of the epigenome in the developing organism in response to the early life environment [141]. Four previously defined human insulin-like growth factor 2 (*IGF2*)*/H19* differentially methylated regions (DMR) were examined in numerous cell types, including cord blood

mononuclear cells, granulocytes, human umbilical vein endothelial cells, buccal epithelial cells, and placenta, from 56 monozygotic and 25 dizygotic newborn twin pairs. The results demonstrated a large amount of variation in DNA methylation between tissues and between unrelated individuals; however, methylation discordance was also present within twin pairs, suggesting that the intrauterine period is a sensitive time for the establishment of epigenetic variability in humans.

Epigenetics has typically been defined as the study of heritable changes in gene expression that occur independent of changes in the primary DNA sequence, while genetics consists of information transmitted on the basis of gene sequence [142, 143]. Recently, however, the term 'epigenetics' has been redefined as the study of heritable changes in gene expression potential [113]. This definition highlights the fact that cell-specific gene expression is not cell-autonomous, and that instead, it responds to various extracellular signals such as paracrine, endocrine, and nutrient signals. Epigenetic modifications consist of DNA methylation, covalent histone modifications, non-covalent mechanisms such as incorporation of histone variants and nucleosome remodelling, and non-coding RNAs including microRNAs [143]. For the purpose of this literature review, the main focus will be DNA methylation.

In the mammalian genome, DNA methylation involves the covalent post-replicative addition of a methyl group onto the 5^{th} carbon of a cytosine base located within a CpG dinucleotide sequence [144]. Therefore, the term 'CpG' includes both methylated and unmethylated nucleotides, with the "p" referring to the phosphate moiety connecting deoxycytidine and deoxyguanosine [145].

CpG dinucleotides are statistically predicted to occur with a frequency of approximately 6% in the human genome; however, their actual presence is estimated to be only 5% to 10% of their predicted frequency [146]. This CpG suppression is most likely due to the fact that methylated cytosines can spontaneously deaminate to form thymine, and since this is not efficiently recognized by DNA repair machinery, C-T mutations accumulate during evolution, resulting in approximately 99% of the human genome being CpG depleted [145]. However, despite this CpG deficiency in vast stretches of the DNA sequence, such as the intergenic and the intronic regions, these regions are interspersed by CpG-rich regions, commonly referred to as "CpG islands" [142]. Typically, CpG islands are defined as being longer than 500 base pairs, having a GC content of at least 55%, and an observed CpG/expected CpG ratio of 0.65. These motifs span the 5' end of approximately half of the human genes, including the promoter, untranslated region, and exon 1.

In the human genome, approximately 70 to 80% of all CpG sites are normally methylated [142]. The majority of this methylation, however, occurs in the bulk of the genome where CpG dinucleotide density is low, including exons, noncoding regions, and repeat DNA sites, and allows correct organization of chromatin in active and inactive states (**Figure 2.4**). Methylation of the CpG-depleted bulk of the genome is important as it facilitates the transcriptional silencing of noncoding regions, therefore preventing the transcription of repeat DNA elements, inserted viral sequences, and transposons [147].

In contrast, CpG islands, which make up approximately 1% of the genome, typically remain unmethylated during development and in differentiated tissues, allowing transcription to occur (**Figure 2.4**) [148]. Under certain circumstances, however, methylation of CpG islands may occur and result in heritable silencing of transcription of downstream genes. In

contrast to methylated CpG sites in the CpG-poor bulk of the genome and unmethylated CpG islands in normal cells, cancer cells simultaneously harbor widespread loss of methylation in the CpG-depleted regions where most CpG dinucleotides should be methylated and gains in methylation of CpG islands in gene promoter regions.

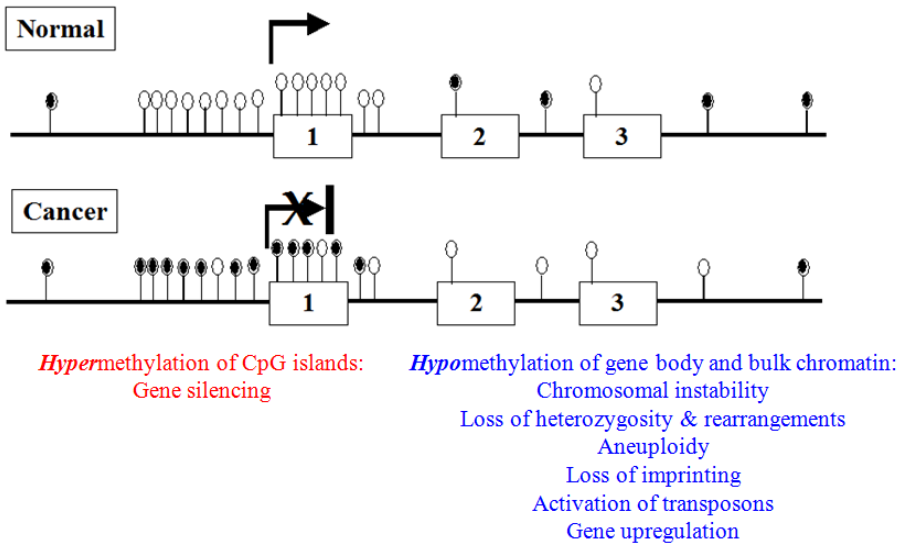

Figure 2.4 Distribution of CpG dinucleotides throughout the human genome and CpG methylation patterns in normal and tumor cells. In normal cells, CpG sites in the CpG-poor bulk of the genome are typically methylated while CpG islands are typically unmethylated. In contrast, cancer cells simultaneously harbor widespread loss of methylation in the CpG-depleted regions where most CpG dinucleotides should be methylated and gains in methylation of CpG islands in gene promoter regions. Open circles represent unmethylated CpG sites whereas filled circles are methylated CpG sites. Boxes 1, 2 and 3 represent exons and lines between exons are introns. X at the transcription start site represents transcriptional silencing [2].

X chromosome inactivation and imprinted genes are two examples of naturally occurring CpG island methylation during development which result in monoallelic gene expression [143]. During embryogenesis, X chromosome inactivation results in the silencing

of one of the two female X chromosomes, in order to equalize the level of expression of X-linked genes in both sexes [149]. Genomic imprinting differs from the vast majority of autosomal genes that are expressed from both parentally contributed alleles, in the sense that one of the two parental alleles are silenced [150]. Approximately 100 to 200 genes within the human genome have been suggested to be subject to genomic imprinting; however, determining an exact number is difficult to quantify as monoallelic expression of imprinted genes may occur in one specific isoform, only in specific tissue types, or only during a specific stage of development [151]. At present, over 80 imprinted genes have been identified in mammals.

A family of enzymes referred to as DNA methyltransferases (DNMTs) are responsible for the transfer of a methyl group from SAM to the cytosine base [152]. At present, several DNMTs have been identified: DNMT1, DNMT2, DNMT3a, and DNMT3b. With the exception of DNMT2, which methylates cytosine 38 in the anticodon loop of tRNA, all other DNMTs appear to be critical for embryonic viability [152, 153]. DNMT3a and DNMT3b are *de novo* methyltransferases, involved in the methylation of previously unmethylated CpG sequences [154]. *De novo* methylation plays a critical role in the establishment of methylation patterns in the early embryo, during development, and during carcinogenesis. DNMT1, on the other hand, is localized to the replication fork during the synthesis phase of the cell cycle and functions as a maintenance DNMT, involved in methylating the newly synthesized DNA strand using the parent strand as a template. Thus, DNMT1 is critical for passing the epigenetic information through cell generations [152].

2.2.4.1.1 Analyzing DNA Methylation: Various Approaches

At present, there are numerous techniques to determine the extent and patterns of DNA methylation at the genomic, gene-specific, and site-specific levels. High-performance liquid chromatography (HPLC) and related techniques, immunostaining, the *in vitro* methyl acceptance capacity assay, and the cytosine extension assay are common methods used to detect genome-wide and global DNA methylation content [155]. It is important to note that there are disadvantages associated with each technique. For example, HPLC tends to be time consuming and laborious, and requires amounts of DNA that may be limiting in some circumstances. In addition, the *in vitro* methyl acceptance capacity assay appears to have a considerable amount of day-to-day variation and difficulties of dissolving genomic DNA to homogeneity can lead to inaccurate DNA concentration measurements.

In contrast to analyzing genomic DNA methylation content which can be determined directly on crude DNA preparations, analyzing gene-specific DNA methylation patterns requires initial amplification of the target sequence [155]. There are numerous enzymes and chemicals available for covalent base modification to allow discrimination between cytosine and 5-methylcytosine, such as methylation-sensitive restriction endonucleases, bisulfite, hydrazine, and permanganate. When measuring the methylation status of specific genes, DNA is often digested with methylation-sensitive restriction enzymes followed by Southern blotting or polymerase chain reaction (PCR)-based semiquantitative analysis [156]. Although reliable, this method requires a substantial amount of high-quality DNA. The use of postdigestion PCR to avoid these limitations is prone to false-positive results due to incomplete enzyme cleavage. In addition, another limitation of this DNA methylation assay

is the number of CpG dinucleotides that can be assessed, as only those CpG sites in the recognition sequences for specific methylation-sensitive restriction enzymes can be analyzed.

Most current methods rely on treatment of genomic DNA with bisulfite prior to PCR amplification (based on the conversion of unmethylated but not methylated cytosines to uracil), including methylation-specific PCR, methylation-sensitive single-nucleotide primer extension, bisulfite genomic sequencing, MethyLight, and methylation-specific single-strand conformation analysis [155, 156]. However, there are certain drawbacks for these methods. For example, information obtained by methylation-specific PCR should only be considered qualitative; thus, the results should be further validated using a quantitative or semiquantitative method, and in addition, often only a single CpG site is reliably probed [155].

More recently, however, high-throughput methods have made it possible to simultaneously analyze the methylation status of thousands of CpG islands in a pool of DNA using such techniques as restriction landmark genomic scanning (RLGS), methylated CpG island amplification-representational difference analysis, differential methylation hybridization, and methylation-sensitive arbitrarily primed PCR [155]. However, there are numerous limitations with these methods. For example, even small amounts of degraded DNA used in RLGS can result in a significant reduction in the quality of the RLGS profiles [155]. Furthermore, while the technique is beneficial for simultaneously assessing the methylation status of numerous CpG sites, it does not provide information to indicate whether or not a CpG island is present within the promoter region of a gene.

2.2.4.1.2 DNA Methylation and Gene Expression

DNA methylation is one type of epigenetic modification that controls and modulates gene expression through chromatin structure [152]. Typically, methylation of CpG dinucleotides occurs in close proximity to important *cis*-elements within promoters and is associated with a repressed chromatin state and the inhibition of transcription [152]. However, an unmethylated CpG island does not necessarily correlate with the transcriptional activity of the gene. In addition, methylation does not necessarily result in the silencing of nearby genes. Often, the expression of a gene will only be modified when a specific core region of the promoter that spans the transcription start site is hypermethylated. Thus, the status of methylated CpG dinucleotides in the core region may better correlate with the expression of the associated gene compared to the overall methylation level of the entire CpG island.

There are several different mechanisms by which DNA methylation can interfere with transcription [152]. The first mechanism involves methylated CpG dinucleotides directly inhibiting the binding of transcriptional activators to their cognate DNA recognition sequence. Secondly, DNA-methyl-binding domain proteins (MBDs) and DNMTs may bind to CpG dinucleotides that are methylated and therefore prevent the binding of potentially activating transcription factors. In addition, MBDs and DNMTs can recruit additional proteins with repressive function, including histone deacetylases, to establish a repressive chromatin configuration [152]. At methylated sites, protein complexes with multiple repressive proteins have been isolated, indicating that gene silencing may require multiple layers for efficient repression, not by one single mechanism [157].

2.2.4.1.3 DNA Methylation during Development

Following cell differentiation or upon exiting the cell cycle, epigenetic marks become fixed for most cells in the body [20]. However, there are certain developmental stages and certain disease states such as cancer, where epigenetic marks may be removed or modified - a process known as epigenetic 'reprogramming'. During mammalian development, DNA methylation is critical, and reprogramming of DNA methylation patterns in the mammalian genome occurs in two distinct phases: in the early preimplantation embryo and during gametogenesis.

When fertilization occurs, the sperm initially appears to have much higher levels of DNA methylation compared to the oocyte [158]. However, within several hours, DNA methylation in the maternal genome rapidly increases [157]. In addition, following histone acquisition of the paternal genome, a genome-wide loss of DNA methylation occurs prior to the first replication event – a process termed 'active demethylation' [159]. The maternal genome remains methylated and may even undergo further *de novo* methylation; however, shortly thereafter, DNA methylation decreases in a step-wise manner over several DNA replication cycles until the morula stage [160]. This second decrease in DNA methylation is partly due to the fact that DNMT1 is retained in the cytoplasm of preimplantation embryos and therefore, the newly replicated strand fails to become methylated and the level of methyl cytosine per nucleus declines - a process referred to as 'passive demethylation' [159]. Demethylation removes most of the pre-existing patterns of methylation in approximately equal amounts from both of the parental genomes [157]. Several studies have determined that repetitive elements including long interspersed nuclear element-1 (LINE-1) and minor satellites become demethylated prior to implantation, as well as tissue-specific and

housekeeping genes, while the paternally imprinted genes are not demethylated. During the second wave of demethylation primarily on the maternal genome, different sequence classes seem to be affected, while the control regions of imprinted genes do not become demethylated. Furthermore, intracisternal A particles (IAP) repetitive elements appear to lose only minimal methylation levels.

Around the time of implantation, cell lines begin to commit to different developmental lineages, and *de novo* methylation in the embryo occurs, restoring DNA methylation levels [159]. The increase in DNA methylation occurs at a variety of gene sequences, with the exception of CpG islands that have a high CpG content but low DNA methylation. At this time, two cell lineages are established: the inner cell mass (ICM) and the trophectoderm (TE) [160]. Both cell lineages gradually gain DNA methylation; however, the ICM appears to be more methylated than the TE, and this is maintained throughout pregnancy [159].

A second reprogramming event occurs during embryogenesis; however, this event occurs only in the primordial germline. Primordial germ cells, derived from the ICM of the developing embryo, arise in the posterior primitive streak at the base of the allantois on embryonic day 7.5 and appear to be initially highly methylated [160]. Upon migration to the genital ridge, a decrease in methylation occurs, with the exception of imprinted genes. However, shortly thereafter, these imprinted genes undergo a rapid demethylation wave, erasing the parental contribution in primordial germ cells, and by embryonic day 12.5, most sequences have become maximally demethylated. Imprinting marks are replaced with new signals consistent with the gender of the developing embryo, and gametogenesis is halted [151]. The timing of remethylation appears to be sex-specific, as remethylation appears to

begin prior to birth at the prospermatgonia stage (embryonic day 15 and onwards) in males, while remethylation in the female germ line takes place during the growth of oocytes following birth [161].

2.2.4.1.4 Folate and DNA Methylation

In addition to its essential role in DNA synthesis, folate also plays a critical role in the provision of methyl groups for the conversion of homocysteine to methionine, which has important functions in methylation reactions including that of DNA (**Figure 2.5**). The enzyme MTHFR is responsible for the NADPH-dependent reduction of 5,10-methyleneTHF, an intracellular co-enzymatic form of folate, into 5-methylTHF [162]. Folate, in the form of 5-methylTHF, will then serve as a methyl group donor in the conversion of homocysteine to methionine [162]. Methionine, activated by adenosine-5-triphosphate (ATP), subsequently forms the methyl cofactor SAM, a versatile coenzyme and a methyl group donor for the majority of biological methylation reactions, including that of DNA. The end result of this reaction is the production of *S*-adenosylhomocysteine (SAH) [162]. SAH is then hydrolyzed to homocysteine, which is then either catabolized or remethylated to methionine.

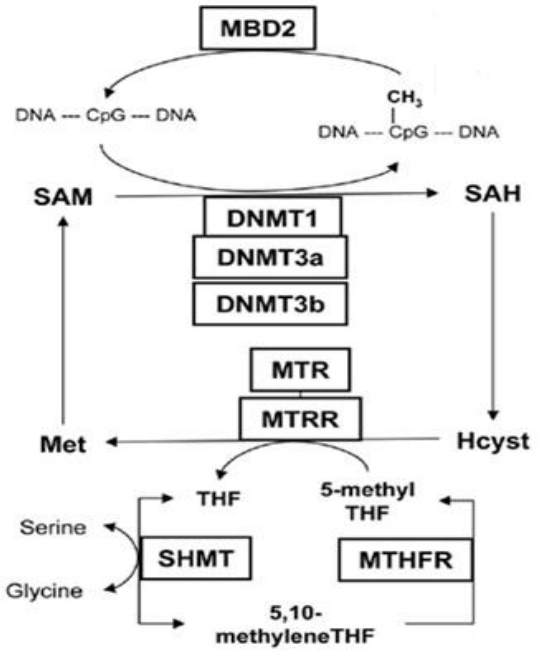

Figure 2.5 Simplified scheme of the role of folate in DNA methylation. MBD2, DNA demethylase; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; DNMT, DNA methyltransferase; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; Hcyst; homocysteine; Met, methionine; SHMT, serine hydroxymethyltransferase; THF,tetrahydrofolate. Modified from [2].

2.2.4.1.4.1 Effects of Folate Deficiency on DNA Methylation: Evidence from Animal Studies

Among the various animal studies that have investigated the effect of isolated folate deficiency on genomic and gene-specific DNA methylation patterns, the liver and colon have been most extensively studied (**Table 2.5**). In rat liver, multiple studies have demonstrated that isolated folate deficiency is capable of reducing SAM concentrations and SAM:SAH concentration ratios, and increasing SAH levels [18, 163-165]. However, the effect of isolated folate deficiency on DNA methylation in rodent liver is less clear.

Few studies have examined the effect of severe folate deficiency for a relatively short duration on DNA methylation [18, 166]. A moderate degree of dietary folate deficiency for a prolonged duration initiated at weaning or later in life has been shown to decrease hepatic SAM concentrations, increase hepatic SAH concentrations, and increase SAM:SAH concentration ratios, but failed to alter hepatic genomic DNA methylation [163, 164, 167]. Interestingly, however, in elder rats (12 months of age), folate deficiency of a moderate degree for 8 and 20 weeks demonstrated significantly decreased hepatic DNA methylation compared to elder rats that consumed a folate-supplemented diet [165]. Folate deficiency of a moderate degree for a shorter duration has also been shown to have no effect on hepatic genomic DNA methylation in pregnant rats as well as male rats [168, 169]. On the other hand, a moderate degree of folate deficiency in mice for 5 weeks' duration was shown to induce a significant 56% increase in hepatic genomic DNA methylation, which returned to that of the baseline by 8 weeks [170].

The effects of both timing and duration of dietary folate intervention on liver genomic DNA methylation were recently examined in a study by Kotsopoulos *et al.* [19]. Interestingly, folate deficiency of a moderate degree beginning either at 3 or 8 weeks of age until 30 weeks of age, failed to modulate genomic DNA methylation in adult rat liver, while the same degree of dietary folate deficiency beginning 3 weeks of age, continuing through early infancy and childhood until 8 weeks, followed by the control diet for 22 weeks, induced a significant 34-48% increase in hepatic genomic DNA methylation [19].

The effect of isolated folate deficiency on gene-specific methylation in the liver has also been examined. In rat liver, severe folate deficiency has been shown to produce

significant hypomethylation within mutation hot spot (exons 6-7) but not in exon 8, of tumor protein 53 (*p53*), despite a 56% increase in genomic DNA methylation [18].

The rodent colon appears to be more resistant to changes in genomic DNA methylation than the rodent liver. A prolonged, moderate degree of dietary folate deficiency in weanling rats failed to alter SAM and SAH concentrations as well as genomic and *c-myc*-specific DNA methylation [163]. In another study, this same degree of dietary folate deficiency significantly increased SAH concentrations; however, genomic DNA methylation in the colon was not altered [164]. While several other studies did not measure colonic SAM and SAH concentrations, a moderate degree of dietary folate deficiency for a prolonged period was shown to have no effect on colonic genomic DNA methylation [167, 171, 172]. In addition, studies examining a moderate degree of folate deficiency in conjunction with the alkylating colon carcinogen dimethylhydrazine (DMH) or with azoxymethane (AOM) have generally failed to alter genomic DNA methylation in rat colon [173-176]. However, it is important to note that DMH and AOM may alter tissue SAM and SAH levels and the extent of DNA methylation, independent of the effect of folate. In contrast to these studies, severe folate deficiency failed to alter genomic DNA methylation in the colon, despite a marked reduction in plasma and colon folate concentrations, an increase in plasma homocysteine, and a progressive decrease in the colon SAM:SAH concentration ratio, while isolated folate deficiency significantly increased the extent of genomic DNA methylation in the colon compared to the control and folate-supplemented groups at an intermediate time point [25].

Although the colon appears to be generally resistant to changes in genomic DNA methylation in response to a moderate degree of folate deficiency, a recent study found that chronic, severe folate deficiency in adult mice induced significant genomic DNA

hypomethylation in the colon, as well as a non-significant degree of genomic DNA hypomethylation in the small intestine and spleen [177]. However, it is important to note that the mice utilized in this study were of older age and therefore may have been more vulnerable to changes in DNA methylation compared to younger mice [177].

In the study by Sohn *et al*., the methylation status of the *p53* promoter and exons 6-7 varied considerably at each of the CpG sites examined over time [25]. In adult mice, dietary folate deficiency of a moderate degree for 10 weeks induced a minor, nonsignificant degree of colonic hypomethylation in exon 6 of the *p53* gene; however, this was amplified by multiple B-vitamin depletion [178]. Furthermore, this same group found that the methylation status of 3 CpG islands in the adenomatous polyposis coli (*Apc*) gene was not altered following folate deficiency [172]. In another study, significant *p53* hypomethylation in exon 8, but not in exons 6-7, was observed in the DMH-treated rat colon in conjunction with folate deficiency [179].

Table 2.5 Effects of folate deficiency on DNA methylation in animal studies

Study (Reference)	Folate Deficiency	Age Diet Began	Duration of Diet	Species (Strain)	Organ/ Tissue	DNA Methylation	Effect
Balaghi and Wagner 1993 [166]	Severe	3 weeks	4 weeks	Rat (Sprague-Dawley)	Liver	Genomic	20% decrease (p=0.032)
Kim *et al*. 1997 [18]	Severe	3 weeks	6 weeks	Rat (Sprague-Dawley)	Liver	Genomic	60% increase (p=0.1)
						p53 (exons 6-7)	40% decrease (p=0.002)
Kim *et al*. 1995 [163]	Mild	3 weeks	15 & 24 weeks	Rat (Sprague-Dawley)	Liver	Genomic	No effect
					Colon	Genomic	No effect
						c-myc	No effect
Uthus *et al*. 2006 [164]	Mild	3 weeks	10 weeks	Rat (Fischer-344)	Liver	Genomic	No effect
					Colon		No effect
Duthie *et al*. 2010 [167]	Mild	6-7 weeks	24 weeks	Rat (Hooded-Lister)	Liver	Genomic	No effect
					Colon		No effect
Choi *et al*. 2005 [165]	Mild	1 year	8 & 20 weeks	Rat (Sprague-Dawley)	Liver	Genomic	Decrease in deficient group compared to supplemented group (p<0.05)
Maloney *et al*. 2007 [168]	Mild	8-10 weeks	~5 weeks	Rat (Rowett Hooded)	Liver	Genomic	No effect
Partearroyo *et al*. 2010 [169]	Mild	5 weeks	30 days	Rat (Sprague-Dawley)	Liver	Genomic	No effect
Song *et al*. 2000 [170]	Mild	3 weeks	8 weeks	Mouse (C57BL/6)	Liver	Genomic	No effect
		6 weeks	5 weeks				56% increase (p<0.05)
Kotsopoulos *et al*. 2008 [19]	Mild	3 weeks	27 weeks	Rat (Sprague-Dawley)	Liver	Genomic	No effect
		8 weeks	22 weeks				No effect
		3 weeks	5 weeks followed by control for 22 weeks				34-48% increase at 30 weeks of age (p<0.04)
Duthie *et al*. 2000 [171]	Mild	6-7 weeks	10 weeks	Rat (Hooded-	Colon	Genomic	No effect

				Lister)			
Liu *et al*. 2007 [172]	Mild	4 months	10 weeks	Mouse (C57BL/6)	Colon	Genomic	No effect
						3 CpG islands of *Apc* (1 in promoter and 2 in intron 1)	No effect
Kim *et al*. 1996 [173]	Mild + DMH	3 weeks	20 weeks	Rat (Sprague-Dawley)	Colon	Genomic	No effect
Le Leu *et al*. 2000	Mild + AOM	4 weeks	12 weeks	Rat (Sprague-Dawley)	Liver	Genomic	No effect
					Colon		No effect
Le Leu *et al*. 2000	Mild + AOM	3 weeks	26 weeks	Rat (Sprague-Dawley)	Colon	Genomic	No effect
Davis and Uthus 2003 [176]	Mild + DMH	3 weeks	12 weeks	Rat (Fischer-344)	Liver	Genomic	No effect
					Colon		No effect
Linhart *et al*. 2009 [177]	Severe	3 months	32 weeks	Mouse (C57BL/6)	Colon	Genomic	6% decrease ($p<0.04$)
					Small intestine		No effect
					Spleen		No effect
Sohn *et al*. 2003 [25]	Severe	3 weeks	5 weeks	Rat (Sprague-Dawley)	Colon	Genomic	30% increase at week 3 ($p=0.022$) No effect at other time points
						p53 (promoter & exons 6-7)	Highly variable; decrease at CpG site 1 in exons 6-7 at week 5
Liu *et al*. 2008 [178]	Mild	4 months	10 weeks	Mouse (C57BL/6)	Colon	*p53* (exons 5-8)	No effect
Kim *et al*. 1996 [179]	Mild + DMH	3 weeks	20 weeks	Rat (Sprague-Dawley)	Colon	*p53* (exon 8)	25% decrease ($p=0.038$)

**p53*=tumor protein 53; *c-myc*=v-myc myelocytomatosis viral oncogene homolog; CpG=cytosine-guanine dinucleotide sequence; *Apc*=adenomatous polyposis coli; DMH=dimethylhydrazine; AOM=azoxymethane

2.2.4.1.4.2 Effects of Folate Supplementation on DNA Methylation: Evidence from Animal Studies

In contrast to folate deficiency, few animal studies have examined the effect of isolated folate supplementation on DNA methylation (**Table 2.6**). Dietary folate supplementation at 4 times the basal dietary requirement of the rat (8 mg folic acid/kg diet) and at supranormal doses initiated at weaning or later in life have failed to modulate genomic DNA methylation in rodent liver [19, 169, 180]. In contrast, elder rats fed a folate-supplemented diet (18 μmol/kg diet) for 8 and 20 weeks demonstrated an increase in hepatic genomic DNA methylation compared to folate-deficient rats [165].

In the colon, DMH administration in conjunction with folate supplementation (8 mg folic acid/kg diet and 40 mg folic acid/kg diet) for 20 weeks in weanling rats did not alter concentrations of SAM and SAH, SAM:SAH concentration ratios, and genomic DNA methylation [173]. Furthermore, in weanling rats fed a folate-supplemented diet (8 mg folic acid/kg diet) for 5 weeks, colonic concentrations of SAM, SAH, SAM:SAH concentration ratios, and colonic genomic and *p53*-specific DNA methylation were not altered [25]. In addition, genomic DNA methylation in the colon of both young and elder rats that consumed a folate-supplemented diet (18 μmol/kg diet) for 8 and 20 weeks was not affected, despite a decrease in colon SAH concentrations [181]. However, in elderly mice fed a folate-deficient, -replete, or –supplemented diet (0 μmol/kg diet, 4.5 μmol/kg diet, or 18 μmol/kg diet, respectively), genomic and cyclin-dependent kinase inhibitor 2A (*p16*)-specific DNA methylation increased in a manner that was directly related to dietary folate, while these effects were not observed in young adult mice [182].

Table 2.6 Effects of folate supplementation on DNA methylation in animal studies

Study (Reference)	Folate Supplementation	Age Diet Began	Duration of Diet	Species (Strain)	Organ/ Tissue	DNA Methylation	Effect
Kotsopolous *et al*. 2008 [19]	8 mg folic acid/kg diet	3 weeks	27 weeks	Rat (Sprague-Dawley)	Liver	Genomic	No effect
		8 weeks	22 weeks				No effect
		3 weeks	5 weeks followed by control for 22 weeks				No effect
Achon *et al*. 2007 [180]	40 mg folic acid/kg diet	3 weeks	4 weeks	Rat (Wistar)	Liver	Genomic	No effect
Partearroyo *et al*. 2010 [169]	8 mg folic acid/kg diet	5 weeks	30 days	Rat (Sprague-Dawley)	Liver	Genomic	No effect
	40 mg folic acid/kg diet						No effect
Choi *et al*. 2005 [165]	18 µmol folate/kg diet	1 year	8 & 20 weeks	Rat (Sprague-Dawley)	Liver	Genomic	Increase in supplemented group compared to deficient group ($p<0.05$)
Kim *et al*. 1996 [173]	8 mg folic acid/kg diet	3 weeks	20 weeks	Rat (Sprague-Dawley)	Colon	Genomic	No effect
	40 mg folic acid/kg diet + DMH						No effect
Sohn *et al*. 2003 [25]	8 mg folic acid/kg diet	3 weeks	5 weeks	Rat (Sprague-Dawley)	Colon	Genomic	No effect
						p53	No effect
Choi *et al*. 2003 [181]	18 µmol folate/kg diet	3 weeks	8 weeks & 20 weeks	Rat (Sprague-Dawley)	Colon	Genomic	No effect
		1 year	8 weeks & 20 weeks				No effect
Keyes *et al*. 2007 [182]	18 µmol folate/kg diet	4 months	20 weeks	Mouse (C57BL/6)	Colon	Genomic	No effect
						p16	No effect
		18 months				Genomic	Stepwise increase from folate-deplete, -replete, and -supplemented ($p_{trend}<0.023$)
						p16	Stepwise increase from folate-deplete, -replete, and -supplemented ($p_{trend}=0.009$)

*DMH=dimethylhydrazine; *p53*=tumor protein 53; *p16*=cyclin-dependent kinase inhibitor 2A

2.2.4.1.4.3 Effects of Folate Deficiency and Supplementation on DNA Methylation: Evidence from In Vitro Studies

The effect of folate deficiency and supplementation on DNA methylation has also been examined in several *in vitro* studies (**Table 2.7**). In normal human colonic epithelial cells cultured in folate-deficient (>2.3 nmol/L) medium for 14 days, a decrease in genomic DNA methylation, increase in uracil misincorporation, and inhibition of DNA excision repair were observed compared to cells cultured in the control (9.1 μmol/L folic acid) medium [183]. In another study using a human colonic adenocarcinoma cell line, cells grown in a folate-free medium for 7 days demonstrated genomic DNA hypomethylation, and by 14 days of folate-depletion, this decrease in genomic DNA methylation was statistically significant [184]. Similarly, cells grown in the folate-free medium displayed significant *p53*-specific hypomethylation by day 7; however, folate depletion beyond this time point did not further increase the severity of hypomethylation [184]. When folic acid was reintroduced in the cells, genomic and *p53* gene-specific DNA methylation levels returned to normal [184].

In a study using untransformed NIH/3T3 mouse fibroblast cells and CHO-K1 Chinese hamster ovary cells, folate deficiency (0.6 nmol/L) was shown to induce significant genomic DNA hypomethylation in both cell lines, by cell-specific mechanisms as indicated by cell-specific differential effects of folate deficiency on intracellular SAM, SAH, and DNMT, while a similar experiment using two human colon adenocarcinoma cell lines demonstrated that the extent and the direction of the changes in SAM and SAH in response to folate deficiency (0.6 nmol/L folic acid) are cell-specific, and that genomic-, site- and gene-specific DNA methylation were not altered, despite changes in SAM and SAH concentrations [185]. Furthermore, in a recent study using three cell lines derived from the

normal human colon grown for 32-34 days in media containing 25, 50, 75, or 150 nmol/L folic acid, folate status failed to alter genomic DNA methylation in any of the cell lines [186].

In another study, hypermethylation in a 5' CpG island of the H-cadherin (*CDH13*) gene was observed in human nasopharyngeal carcinoma cells grown in a folate-deplete (2-10 nmol/L folic acid) medium compared to cells grown in a folate-replete (2.0 μmol/L folic acid) medium [27].

In a recent study using three syngenic but phenotypically distinct clonal cell lines derived from the transgenic adenoma of a mouse prostate model, recapitulating different stages of prostate cancer (benign, tumorigenic, metastatic), long-term (20 population doublings) mild folate depletion (100 nM folic acid) did not alter genomic DNA methylation in any of the cells despite an observed imbalance in both the nucleotide and SAM pools, a significant increase in uracil misincorporation and DNA single strand breaks, and five novel chromosomal rearrangements in cells grown with mild folate depletion [187]. However, using RGLS to measure global CpG island methylation, 14 hypermethylated loci within numerous genes including insulin-like growth factor 2 mRNA binding protein 3 (*Igf2bp3*), proline dehydrogenase (oxidase) 1 (*Prodh*), mitogen-activated protein kinase 4 (*Mapk4*), and cyclin M1 (*Cnnm1*) were observed, and the most profound changes were observed in tumorigenic cells.

Table 2.7 Effects of folate deficiency and supplementation on DNA methylation in *in vitro* systems

Study (Reference)	Cell Type	Folate Level	Duration of Folate Deficiency or Supplementation	DNA Methylation	Effect
Duthie *et al*. 2000 [183]	Normal adult colon cells immortalized using SV40 T antigen	Folate deplete (<1 ng/mL folic acid) vs. (4 mg/L folic acid)	14 days	Genomic	~ 40% decrease ($p<0.5$)
Wasson *et al*. 2006 [184]	Established human colonic adenocarcinoma cell lines SW620	Folate free vs. normal amounts (3 µmol/L folic acid)	7 or 14 days	Genomic	Decrease by day 14 ($p=0.014$)
Stepmak *et al*. 2005 [185]	Untransformed NIH/3T3 mouse fibroblast cells	Folate deficient (0 µM folic acid) vs. control (2.3 µM folic acid)	12 days	Genomic	~ 20% decrease ($p=0.003$)
				Satellite	Decrease in a mouse centromeric minor satellite repeat sequence
	Untransformed CHO-K1 Chinese hamster ovary cells		12 days	Genomic	~ 20% decrease ($p=0.002$)
	Human colon adenocarcinoma cells HCT116		20 days	Genomic	No effect
				Satellite	No effect
				ER, *p16*, *MLH1* gene promoters	~ 25% increase at CpG site 10 of *ER* ($p<0.05$)
	Human colon adenocarcinoma cells Caco-2 cells		20 days	Genomic	No effect
				Satellite	No effect
				ER, *p16*, *MLH1* gene promoters	~ 20% increase at site CpG 2 and 25% decrease at CpG sites 6 and 11 of *ER*
Crott *et al*. 2008 [186]	3 colonic epithelial lines derived from normal adult human colon, SV40 transformed	Increasing levels: 25, 50, 75, or 150 nM folic acid	30-32 days	Genomic	No effect
Jhaveri *et al*. 2001 [27]	Human nasopharyngeal epidermoid carcinoma	Folate deplete (2-10 nmol/L folic acid)		*CDH13*	40% increase

	KB cells	vs. folate replete (2 μmol/L folic acid)			
Bistulfi *et al*. 2010 [187]	3 syngenic prostate cell lines from transgenic adenoma of mouse prostate model (benign, tumorigenic, and metastatic)	Low (100 nM folic acid) vs. control (2 μM folic acid)	Long term (20 population doublings)	Genomic	No effect
				Global CpG island (1200 CpG islands throughout genome)	Increase in numerous genes (including *Igf2bp3*, *Prodh*, *Mapk4*, *Cnnm1*) Most profound changes in transformed cells

**ER*=estrogen receptor; *p16*=cyclin-dependent kinase inhibitor 2A; *MLH1*=mutL homolog 1; *CDH13*=H-cadherin; CpG=cytosine-guanine dinucleotide sequence; *Igf2bp3*=insulin-like growth factor 2 mRNA binding protein 3, *Prodh*=proline dehydrogenase (oxidase) 1; *Mapk4*=mitogen-activated protein kinase 4; *Cnnm1*= cyclin M1

2.2.4.1.4.4 Effects of Folate Status on DNA Methylation in Human Clinical Trials

Human interventional studies have also suggested that altered folate status may modulate genomic DNA methylation, while others have reported no effect (**Table 2.8**). In healthy, postmenopausal women (60-85 years) that consumed a folate deficient diet of a moderate degree (118 μg folate/day) for 7 weeks, a significant 10% decrease in leukocyte DNA hypomethylation was observed [188]. Following folate depletion, a 7-week period of folate repletion with either 200 or 415 μg of folate/day failed to return leukocyte genomic DNA methylation levels to that of baseline, as values remained similar to those following folate depletion. Jacob *et al*. examined lymphocyte DNA methylation in healthy postmenopausal women (49-63 years) housed in a metabolic unit and fed a folate-deplete diet (56-111 μg folate/day) for 9 weeks [189]. In this study, folate depletion resulted in a significant decrease in lymphocyte DNA methylation, and this was reversed in 3 weeks with folate supplementation (285-516 μg folate/day). Interestingly, in an earlier study, this same group found that in healthy adult men (33-46 years) housed in a metabolic unit and fed a diet low in folate (25 μg folate/day) and exogenous methyl groups for 30 days, *in vivo* methylation capacity was not altered [190].

In a more recent study, the influence of the MTHFR C677T polymorphism on genomic leukocyte DNA methylation in young Mexican American women who were consuming controlled folate intakes was examined [191]. The initial 7 weeks of the controlled feeding study consisted of a folate restriction period, where subjects consumed a low folate diet (135 μg/day DFEs). Following folate restriction, 7 weeks of folate treatment occurred in which subjects continued to consume the folate-restricted diet and in addition consumed 156 or 391 μg/day of supplemental folic acid, providing a total folate intake of

400 or 800 μg/day DFEs. Interestingly, a significant interaction between timing of folate treatment and MTHFR C677T polymorphism on DNA methylation was observed, as leukocyte genomic DNA methylation was significantly lower in women with the TT genotype compared to women with the CC or CT genotype at the end of the 14 weeks. In contrast, using the same experimental design, Axume *et al*. examined the influence of ethnicity/race on genomic leukocyte DNA methylation in Caucasian and African American women wild-type for MTHFR C677T, and found that there were no differences in DNA methylation between African American and Caucasian women, and folate intake did not modify genomic leukocyte DNA methylation [192]. In another study, young healthy women with the TT or CC genotype consumed a low folate diet (115 μg/day DFEs) during the first 7 weeks of the study, followed by folate repletion (400 μg/day DFEs) [193]. A trend was reported towards a decrease in leukocyte DNA methylation in folate-deplete women, and interestingly, folate repletion only corrected this decrease in leukocyte DNA methylation in subjects with the TT genotype. Furthermore, in a subgroup of women from this population, *in vivo* analysis of genomic DNA methylation in monocytes, determined by methyldeoxycytidine enrichment following labelled infusions of [$^{13}C_5$]methionine, also revealed that folate-dependent intracellular one-carbon metabolism was suppressed following 7 weeks of folate restriction (115 μg DFE/day); however, this effect was independent of MTHFR genotype [194].

Table 2.8 Effects of folate deficiency on DNA methylation in human clinical trials

Study (Reference)	Subjects	Folate Level	Duration of Folate Deficiency	DNA Methylation	Effect
Rampersaud *et al*. 2000 [188]	Women 60-85 years	118 µg/day	7 weeks	Leukocytes Genomic	10% decrease (p=0.0012)
Jacob *et al*. 1998 [189]	Women 49-63 years	56-111 µg/day	9 weeks	Lymphocytes Genomic	120% decrease (p<0.05)
Jacob *et al*. 1995 [190]	Men	25 µg/day	30 days	Methylation capacity[¶]	No effect
Axume *et al*. 2007 [191]	Women 18-45 years	135 µg/day	7 weeks	Leukocytes Genomic	No effect
		400-800 µg/day	14 weeks		4% decrease (p<0.05)
Axume *et al*. 2007 [192]	Women 18-45 years	135 µg/day	7 weeks	Leukocytes Genomic	No effect
		400-800 µg/day	14 weeks		No effect
Shelnutt *et al*. 2004 [193]	Women 20-30 years	115 µg/day	7 weeks	Leukocytes Genomic	5% decrease (p=0.08)
		400 µg/day	14 weeks		8% increase (p=0.04)
Quinlivan *et al*. 2004 [194]	Women 20-26 years	115 ± 20 µg/day	7 weeks	Monocytes MdC enrichment	40% decrease (p=0.012)

*MdC enrichment=methyldeoxycytidine enrichment following radiolabelled infusions of [$^{13}C_5$]methionine
[¶]Ability to methylate orally administered nicotinamide as detected in the urine as methylated metabolites

Several human intervention studies have also examined the effect of folate supplementation on DNA methylation (**Table 2.9**). Folic acid supplementation at 12.5-25 times the daily requirement for 3-12 months was shown to significantly increase the extent of colonic DNA methylation in subjects with resected colorectal adenoma or cancer [195-197]. In young healthy adults, lymphocyte DNA methylation was not altered following folic acid supplementation of 3.5 times the daily requirement of folate [198]. Similarly, in healthy adult men and women that received 1.2 mg folic acid for 12 weeks, lymphocyte genomic DNA methylation was not altered [199]. Participants from the Aspirin/Folate Poly Prevention Study also demonstrated no significant association between LINE-1 methylation in normal colonic mucosa and folate treatment or circulating levels of folate and homocysteine [200].

In men with hyperhomocysteinemia, uraemia, and pre-existing genomic DNA hypomethylation, folate supplementation with 15 mg methylTHF for 8 weeks was able to restore genomic DNA methylation in lymphocytes to normal levels [29]. Furthermore, in patients with histologically confirmed colorectal adenomas who received a daily supplement of 400 μg folic acid for 10 weeks, lymphocyte genomic DNA methylation increased by 31%, and colonic mucosa genomic DNA methylation increased by 25% [201]. In a study that examined the combined effects of folic acid and vitamin B_{12} (1.25 mg/day) supplementation for 6 months on promoter methylation of tumor suppressor and DNA repair genes often methylated in colorectal cancer, a trend towards a 67% increase in promoter hypermethylation in rectal mucosal biopsies from patients with resected colorectal adenomas was reported in the intervention group; however, this did not reach statistical significance [202].

In a recent study, the effect of chronic folic acid supplementation and withdrawal on genomic DNA methylation was examined in Chinese women of reproductive age not previously exposed to folic acid [203]. Women consumed folic acid supplements containing 100, 400, and 4000 μg/day for 6 months. Following 1 month of folic acid supplementation, genomic DNA methylation was shown to significantly decrease (by 13%). However, genomic DNA methylation did not change further during the remainder of the intervention (at 3 and 6 months). Interestingly, following a 3 month washout period after folic acid supplementation, a further decrease (by 23%) in genomic DNA methylation was observed relative to baseline values.

Table 2.9 Effects of folate supplementation on DNA methylation in human clinical trials

Study (Reference)	Subjects	Folate Level	Duration of Folate Supplementation	DNA Methylation	Effect
Cravo *et al*. 1994 [195]	Patients with colorectal cancer and adenoma	10 mg/day	6 months	Rectal mucosa Genomic	93% increase ($p<0.002$)
Cravo *et al*. 1998 [196]	Patients with colonic adenoma	5 mg/day	3 months	Rectal mucosa Genomic	37% increase in patients with 1 adenoma ($p=0.05$) and no change in those with >1 adenoma
Kim *et al*. 2001 [197]	Patients with colonic adenoma	5 mg/day	6 months	Rectal mucosa Genomic	57% increase ($p=0.001$)
			1 year		No effect
Fenech *et al*. 1998 [198]	Normal subjects	2 mg/day	12 weeks	Lymphocytes Genomic	No effect
Basten *et al*. 2006 [199]	Normal subjects	1.2 mg/day	12 weeks	Lymphocytes Genomic	No effect
Figueriedo *et al*. 2009 [200]	Patients with colonic adenoma	1 mg/day	6-8 years	Colonic mucosa Genomic	No effect
Ingrosso *et al*. 2003 [29]	Uremic patients with hyperhomocysteinemia & pre-existing DNA hypomethylation	15 mg/day 5-methylTHF	8 weeks	Lymphocytes Genomic	Restored to normal levels
Pufulete *et al*. 2005 [201]	Patients with colonic adenoma	400 µg/day	10 weeks	Lymphocytes Genomic	31% increase ($p=0.05$)
				Rectal	25% increase ($p=0.09$)
van den Donk *et al*. 2007 [202]	Patients with colorectal adenoma	5 mg/day	6 months	Rectal biopsies *APC* *p14* *p16* *hMLH1* *O^6-MGMT* *RASSFIA*	67% increase in probability of promoter hypermethylation of all 6 genes ($p=0.08$)
Quinlivan *et al*. 2008 [203]	Women of reproductive age	100-4000 µg/day	1 month	Genomic	13% decrease ($p<0.001$)
		100-4000 µg/day	3 months		No effect
		100-4000	6 months		No effect

		µg/day			
		0 µg/day	9 months		23% decrease ($p<0.0001$)

**APC*=adenomatous polyposis coli; *p14*=protein 14*; p16*=cyclin-dependent kinase inhibitor 2A; *hMLH1*= mutL homolog 1; *O^6-MGMT*=O-6-methylguanine-DNA methyltransferase; *RASSFIA*=ras association domain family 1

2.2.4.1.4.5 Effects of Folate Status on Genomic DNA Methylation in Human Observational Studies

Numerous human observational studies have also examined the effect of folate status on genomic DNA methylation **(Table 2.10)**. Several studies have reported no significant correlations between genomic lymphocyte DNA methylation status and RBC folate or plasma homocysteine concentrations [198, 204]. In addition, other studies have failed to demonstrate a clear association between blood folate status and methylation-related intermediates. In healthy men (24-68 years), serum folate concentrations in the highest quintile were associated with increased RBC levels of SAM and SAH; however, the SAM:SAH concentration ratio was similar in all three quintiles of serum folate [205]. In elderly men and women (60-85 years), SAM and SAH concentrations, as well as the SAM:SAH concentration ratio, were not associated with serum or RBC folate concentrations [206]. In contrast to these observations, a study including men and women previously diagnosed with adenocarcinoma of the colorectum demonstrated diminished folate status and SAM:SAH concentration ratios in malignant tissue compared with normal appearing mucosa [207].

Numerous studies have suggested that genomic DNA methylation in the colon is positively correlated with serum and RBC folate concentrations and negatively associated with plasma homocysteine concentrations in individuals with colonic adenomas and adenocarcinomas and in individuals with no colorectal abnormalities [208-210]. Similarly, several studies have demonstrated a positive relationship between genomic DNA methylation in peripheral blood mononuclear cells and folate status [211, 212]. In one study, genomic DNA methylation in peripheral blood mononuclear cells directly correlated with folate status

and inversely with plasma homocysteine levels [211]. In addition, those individuals with the MTHFR TT genotype exhibited a significantly lower level of genomic DNA methylation compared to those with the CC wild-type genotype; however, this was only observed under conditions of low folate status.

In a study population of participants from the Nurses' Health Study and the Health Professionals Follow-up Study, the association between dietary intake of folate and the risk of genomic hypomethylation was examined in 609 incident CRCs [213]. Interestingly, the risk of genomic DNA hypomethylation, determined by <55% LINE-1 methylation, was 43% lower in subjects with ≥400 μg/day of folate intake compared to those reporting <200 μg/day of folate intake [213]. In another study, an inverse association between leukocyte genomic DNA methylation and adenoma was observed and appeared to be stronger among subjects with low compared to high total folate intake in both prefortification and postfortification periods [214].

Table 2.10 Effects of folate status on genomic DNA methylation in human observational studies

Study (Reference)	Subjects	Design	Folate Level	DNA Methylation	Effect
Fenech *et al*. 1998 [198]	Normal subjects	Cross-sectional	RBC 364- 440 nmol/L (mean)	Lymphocytes Genomic	No effect
Narayanan *et al*. 2004 [204]	Normal subjects	Cross-sectional	Plasma 15-21 nmol/L	Lymphocytes Genomic	No effect
			RBC folate 191-254 nmol/L		
Pufulete *et al*. 2003 [209]	Patients with colon cancer or adenoma	Case-control	Serum 12 vs. 18 nmol/L	Colon mucosa Genomic	26% decrease (p=0.009)
				Leukocytes Genomic	14% decrease (p<0.001)
Al-Ghnaniem *et al*. 2007 [210]	Patients with colon cancer or adenoma	Case-control	Serum 12.3 vs. 17.9 nmol/L	Colon mucosa Genomic	38% decrease (p<0.001)
Pufulete *et al*. 2005 [208]	Patients without colon cancer or adenoma	Cross-sectional	Serum folate 18.6 nmol/L (mean)	Colon mucosa Genomic	r=-0.311 (p=0.01)
			RBC folate 648.1 nmol/L (median)		r=-0.356 (p=0.003)
			Plasma tHcy 9.9 µmol/L (median)		r=0.256 (p=0.04)
Friso *et al*. 2002 [211]	Patients with and without coronary artery disease	Cross-sectional	Plasma folate <12 vs. ≥12 nmol/L	Lymphocytes Genomic	61% decrease (p<0.0001) in subjects with MTHFR 677TT
			RBC folate <1.1 vs. ≥1.1 nmol/g Hb		65% decrease (p<0.0001) in subjects with MTHFR 677TT
Pilsner *et al*. 2007 [212]	Adults chronically exposed to arsenic	Cross-sectional	Plasma folate <9 vs. ≥9 nmol/L	Leukocytes Genomic	3% decrease (p=0.03)
			Plasma folate 8.6 nmol/L (mean)		r=0.12 (p≤0.05)
Schernhammer *et al*. 2010 [213]	Patients with colon cancer	Prospective cohort	Dietary intake ≥400 vs. <200 µg/day	Colon cancer Genomic	43% decrease risk of hypomethylation (p=0.05)
Lim *et al*. 2008 [214]	Women with colonic adenoma (50-79 years)	Case-control	Dietary intake Prefortification: <317 vs. ≥317 µg/1000 kcal/day Postfortification: <413 vs. ≥ 413 DFE/day	Genomic Leukocyte	Methylation inversely associated with colonic adenoma risk, especially in subjects with low folate intake in pre- and post-fortification

*RBC=red blood cell; tHcy=homocysteine; Hb=hemoglobin; DFE=dietary folate equivalents

2.2.4.1.4.6 Effects of Folate Status on Gene-Specific DNA Methylation in Human Observational Studies

In addition to genomic DNA methylation, several human observational studies have investigated the effect of folate status on gene-specific DNA methylation (**Table 2.11**). In tumors, aberrant CpG island methylation is commonly observed and numerous studies have demonstrated that dietary folate intake or blood folate status can modulate promoter CpG methylation in colorectal tumors. In the Netherlands Cohort Study on Diet and Cancer, the relationship between promoter methylation of genes involved and typically hypermethylated in CRC and the intake of folate and alcohol was examined [215]. Although not statistically significant, there was a trend for greater CpG island promoter hypermethylation in all 6 of the examined genes in CRCs derived from patients with low folate/high alcohol intake compared to those CRCs from patients with high folate/low alcohol intake. Nevertheless, the results suggest that folate and alcohol intake may be associated with changes in promoter hypermethylation in CRC. A follow-up analysis in a subcohort of this population observed no effect of isolated dietary folate intake on risk of tumors demonstrating mutL homolog 1 (*MLH1*) hypermethylation [216]. Interestingly, van den Donk *et al*. observed that folate intake interacted with the MTHFR C677T polymorphism to influence CpG promoter methylation of these 6 genes in colorectal adenomas [217]. Among individuals with the MTHFR 677TT genotype, the risk of promoter methylation was inversely related to dietary folate intake, and this was most pronounced for O-6-methylguanine-DNA methyltransferase (O^6-*MGMT*). Similarly, Slattery *et al*. did not observe a significant association between dietary folate and colon tumors showing CpG island methylator phenotype (CIMP), based on CpG island methylation of *p16*, *MLH1*, and amyloid beta A4 precursor protein-binding (*MINT)-1*, *-2*, and *-3* loci [218]. However, in a follow-up study from this same group,

subjects who were heterozygous or homozygous for the MTHFR A1298C genotype in conjunction with low folate and methionine intake and high alcohol use had a greater than 2-fold risk of CIMP-positive tumors compared to subjects with the wild-type genotype and high folate and methionine intake and low alcohol use [219]. In another study, biopsies of normal-appearing colorectal mucosa from subjects with and without colorectal neoplasia were obtained and methylation of 7 CpG sites in the estrogen receptor (*ER)-α* promoter and 13 CpG sites in the *MLH1* promoter were examined [210]. Interestingly, serum and RBC folate had no influence on *ERα* and *MLH1* promoter methylation; however, *ER-α* methylation was negatively correlated with serum vitamin B_{12} and positively correlated with plasma homocysteine in all subjects.

In contrast, a recent study examined the relationship between prediagnostic plasma folate to the risk of CRC with and without the CIMP, and observed a greater risk for CIMP positive colorectal tumors, using promoter methylation of *p16*, *MLH1*, calcium channel (*CACNA1G)*, neurogenin 1 (*NEUROG1*), run-related transcription factor 3 (*RUNX3)*, suppressor of cytokine signalling 1 (*SOCS1*), *IGF2*, and cellular retinoic acid binding protein 1 (*CRABP1*) and markers, in patients with high plasma folate concentrations [220].

Table 2.11 Effects of folate status on gene-specific DNA methylation in human observational studies

Study (Reference)	Subjects	Design	Folate Levels	DNA Methylation	Effect
van Engeland *et al*. 2003 [215]	Patients with colon cancer	Prospective	Dietary intake <215 µg/day and ≥5 g/day alcohol vs. ≥215 µg/day and 0-4 g/day alcohol	Colon cancer *APC* *p14* *p16* *MLH1* *O^6-MGMT RASSFIA*	Increased prevalence for all genes (p>0.05)
de Vogel *et al*. 2008 [216]	Patients with colon cancer	Prospective	Dietary intake 142.4-163.2 vs. 247-279.9 µg/day	Colon cancer *MLH1*	No associations
van den Donk *et al*. 2007 [217]	Patients with colorectal adenoma	Case-control	Dietary intake <183 vs. >212 µg/day	Colorectal adenoma *APC* *p14* *p16* *MLH1* *O^6-MGMT RASSFIA*	Increased with low folate and decreased with high folate in subjects with MTHFR 677TT
Slattery *et al*. 2007 [218]	Patients with colon cancer	Case-control	Dietary intake <135-152 (low), 135-201 (med), >180-201 (high) µg/1000 kcal/day	Colon cancer CIMP	No associations
Curtin *et al*. 2007 [219]	Patients with colon cancer	Case-control	Dietary intake <135-152 (low), 135-201 (med), >180-201 (high) µg/1000 kcal/day	Colon cancer CIMP	Increased in subjects with MTHFR 1228AC/CC in conjunction with low folate and methionine intake and high alcohol use
Al-Ghnaniem *et al*. 2007 [210]	Patients with colon cancer or adenoma	Case-control	Serum 12.3 vs. 17.9 nmol/L	Colon mucosa *ERα* *MLH1*	No associations
van Guelpen *et al*. 2009 [220]	Patients with colon cancer	Case-control	Plasma ≥6.8 vs. <6.8 µmol/L	Colon cancer CIMP	~3-fold increase (p<0.05)

**APC*=adenomatous polyposis coli; *p14*=protein 14*; p16*=cyclin-dependent kinase inhibitor 2A; *MLH1*= mutL homolog 1; *O^6-MGMT*=O-6-methylguanine-DNA methyltransferase; *RASSFIA*=ras association domain family 1; CIMP=CpG island methylator phenotype; ER*α*=estrogen receptor (*ER)-α*

2.2.4.2 Effects of Maternal Folate Status on DNA Methylation in the Offspring

2.2.4.2.1 Effects of Maternal Nutrition (Excluding Folate) on DNA Methylation in the Offspring: Evidence from Animal Studies

A variety of early environmental factors in addition to nutritional factors, such as valproic acid, alcohol, tobacco smoke, and lead, have been suggested to modify DNA methylation patterns in the offspring [221-224]. However, ***Chapter 2.2.4.2.1*** will focus on the influence of early life nutritional factors (excluding diets containing folate), on DNA methylation in the offspring.

The first clear demonstration that a transient environmental stimulus occurring during a critical period of development could result in a permanent phenotypic change via an epigenetic mechanism was in the viable yellow *agouti* (A^{vy}) mouse model. The A^{vy} allele resulted from the insertion of an IAP retrotransposon upstream of the *agouti* gene [225]. The a*gouti* gene is normally expressed only in hair follicles; however, a cryptic promoter in the A^{vy} IAP confers ectopic *agouti* expression, resulting in mice with a yellow coat colour and a tendency for obesity, cancer, diabetes, and a short life. Since A^{vy} is a metastable epiallele, CpG methylation and overall epigenotype at A^{vy} are established stochastically early on in development, and are then maintained throughout life [21]. Therefore, genetically identical A^{vy}/a mice display a wide range of coat colour phenotypes, from yellow (hypomethylated at A^{vy}) to brown, or pseudoagouti (hypermethylated at A^{vy}) [225]. Maternal methyl group supplementation during pregnancy was shown to alter the phenotype of the offspring, resulting in a greater proportion of offspring with psuedoagouti rather than yellow-coloured coats [21]. This shift in coat colour was due to increased systemic A^{vy} hypermethylation in A^{vy}/a offspring [21, 226].

There have been numerous studies that have examined the effect of a variety of maternal nutritional factors (excluding diets containing folate) on DNA methylation patterns in the offspring (**Table 2.12**). Genistein, the principal isoflavone in soy, has recently been shown to induce phenotypic changes in $A^{vy/a}$ offspring via epigenetic mechanisms [23]. A maternal diet supplemented with 250 mg/kg diet of genistein, a level comparable to humans consuming high-soy diets, resulted in increased methylation at 6 CpG sites in the retrotransposon upstream of the transcription start site of the *agouti* gene, shifting the coat colour of offspring toward the pseudoagouti phenotype. Offspring from mice fed a high fat diet during pregnancy and lactation demonstrated an increased preference for sucrose and fat, and differences in genomic and gene-specific promoter DNA methylation in brain regions associated with reward processing were observed [227]. In baboons, maternal global nutrient restriction throughout pregnancy was shown to induce tissue- and age-specific effects on genomic DNA hypomethylation in the offspring [228]. A diet restricted in protein consumed throughout pregnancy induced hypomethylation of CpG islands in the promoter region of several genes, and some of these have been shown to persist into adulthood [31, 229, 230].

Table 2.12 Effects of maternal nutritional factors (excluding folate) on DNA methylation in the offspring in animal studies

Study (Reference)	Species (Strain)	Maternal Diet	Duration of Maternal Diet	Endpoint	Organ/ Tissue	DNA Methylation	Outcome in Offspring
Dolinoy *et al*. 2006 [23]	Mouse (A^{vy})	Genestein supplementation	2 weeks prior to mating, pregnancy, and lactation	PND150	Tail, liver, brain, kidney	9 CpG sites in cryptic promoter of A^{vy} IAP	Increase at sites 4, 5, 6, 7, 8, 9 (p=0.004, p=0.02, p=0.04, p=0.03, p=0.05, p=0.02, respectively)
Vucetic *et al*. 2010 [227]	Mouse (C57BL/6)	High fat	3 months prior to mating, pregnancy, and lactation	PND126-168	Male brain (4 regions: VTA, PFC, NAc, hypothalamus)	Genomic	Decrease (p<0.0001)
						TH, *DAT*, *MOR*, and *PENK* promoters	Decrease in *Dat*, *Mor*, and *Penk* (VTA and NAc) (p<0.05) Decrease in *Mor* (PFC) (p<0.05)
Unterberger *et al*. 2009 [228]	Baboon	Nutrient restriction	GD30-93	GD93	Frontal cortex, heart, kidney, liver	Genomic	Decrease (kidney) (p=0.07)
			GD30-167	GD167			Increase (frontal cortex and kidney) (p<0.05)
Lillycrop *et al*. 2005 [31]	Rat (Wistar)	Protein restriction	Pregnancy	PND34	Liver	*Pparα*, *Pparγ,* and *GR*	Decrease in *Pparα* (p<0.001) and *GR* (p<0.05)
Goyal *et al*. 2010 [229]	Mouse (FVB/NJ)	Protein-restriction	GD10.5-17.5	GD17.5	Brain	*Ace-1* promoter	Decrease (p<0.05)
Burdge *et al*. 2007 [230]	Rat (Wistar)	Protein restriction	Pregnancy	PND80	Liver	*Pparα* and *GR*	Decrease in *Pparα* and *GR*(p<0.05)

*A^{vy}=viable yellow agouti mouse model; PND=postnatal day; CpG=cytosine-guanine dinucleotide sequence; IAP=intracisternal A particle; VTA=ventral tegmental area; PFC=prefrontal cortex; NAc=nucleus accumbens, *TH*=tyrosine hydroxylase; *DAT*=dopamine reuptake transporter; *MOR*=μ-opioid receptor; *PENK*=preproenkephalin, GD=gestational day; *PPAR*=peroxisome proliferator–activated receptor; *GR*=glucocorticoid receptor; *ACE-1*=angiotensin-converting enzyme 1

2.2.4.2.2 Effects of Maternal Nutrition (Excluding Folate) on DNA Methylation in the Offspring: Evidence from Human Studies

Human studies also provide evidence that the epigenome is susceptible to alterations in maternal diet during development, which may persist into adulthood. A recent study examined the effect of periconceptional exposure to famine during the Dutch Hunger Winter on whole blood DNA methylation at a DMR within the *IGF2* gene [231]. *IGF2* is a maternally imprinted gene involved in growth and cell differentiation, and plays a key role in regulating glucose homeostasis, cardiovascular functions, and lipid metabolism. Complete loss of methylation at the DMR within the *IGF2* gene results in biallelic expression, and has been associated with an increased risk of colorectal adenoma and observed in Beckwith-Wiedemann (BWS) syndrome, which is characterized by overgrowth [232]. Interestingly, adult offspring who were exposed to famine during the periconceptional period demonstrated less DNA methylation of the *IGF2* DMR decades later, compared to their unexposed same-sex siblings, while exposure to famine late in gestation was not associated with *IGF2* DMR methylation in adult offspring. This same group found that periconceptional exposure to famine was associated with altered methylation at 6 of the 15 genes involved in metabolic and cardiovascular disease [233].

2.2.4.2.3 Effects of Maternal Folate Status on DNA Methylation in the Offspring

At present, several preliminary studies suggest that maternal folic acid deficiency or supplementation alone or combined with other dietary factors may alter DNA methylation patterns in the developing offspring **(Table 2.13)**. In studies using the A^{vy} mouse model, maternal dietary methyl group (folic acid, vitamin B_{12}, betaine, and choline) supplementation

has been shown to result in increased CpG methylation in the promoter region of the *agouti* gene. Maternal methyl group supplementation shifted the coat colour distribution of the offspring away from the yellow phenotype and toward the pseudoagouti phenotype and increase DNA methylation in the A^{vy} proximal long terminal repeat [226, 234]. Furthermore, Waterland *et al*. demonstrated that maternal methyl group supplementation altered the phenotype of their A^{vy}/a offspring via increasing CpG methylation at each of seven A^{vy} pseudoexon 1A CpG sites [21]. In another study using the A^{vy} mouse model, the effect of maternal exposure to the estrogenic xenobiotic chemical bisphenol A (BPA) alone or combined with methyl donors on DNA methylation in the offspring was examined [235]. Maternal exposure to BPA for two weeks prior to mating and throughout gestation and lactation shifted the coat colour distribution of A^{vy}/y offspring toward the yellow coat colour phenotype. In addition, BPA-exposed offspring demonstrated significantly lower methylation across 9 CpG sites in the cryptic promoter region of the A^{vy} IAP compared to the control offspring. Interestingly, however, was that maternal dietary supplementation with methyl donors (including 4.3 mg of folic acid/kg diet) counteracted the DNA hypomethylating effect of BPA, as CpG methylation at the A^{vy} IAP of offspring from the methyl donor-supplemented group did not significantly differ from that of control offspring. In addition, maternal methyl donor supplementation restored the coat colour distribution in BPA-exposed offspring to that observed in offspring from the control group.

Similar effects have been observed in the $Axin^{Fu}$ mouse model [22]. The $Axin^{Fu}$ metastable epiallele is the result of an IAP retrotransposon insertion into intron 6 of *Axin*. The $Axin^{Fu}$ IAP induces a downstream cryptic promoter that drives the expression of a biologically active 3' truncated transcript of *Axin.* Spontaneous variability in CpG

methylation at *Axin*^{Fu} results in a large variation of tail kink phenotypes among genetically identical *Axin*^{Fu}/+ mice. Mice with severely kinked tails have complete demethylation of the IAP site within this locus, while those with straight tails have complete methylation of the IAP site [225]. Waterland *et al*. demonstrated that methyl group supplementation before and during pregnancy induced hypermethylation at the *Axin*^{Fu} locus in the offspring, ultimately reducing the incidence of tail kinking in *Axin*^{Fu}/+ offspring [22].

In addition to the A^{vy} and *Axin*^{Fu} mouse models, a variety of animal studies have now demonstrated that alterations in maternal folate status, most often in addition to other dietary components, during the earliest stages of life can alter DNA methylation patterns in the developing offspring. In Scottish Blackface ewes, Sinclair *et al*. demonstrated that adult offspring from mothers on a diet restricted in dietary folate, vitamin B_{12}, and methionine from 8 weeks before until 6 days following conception were born with increased adiposity, insulin resistance, altered immune function, and high blood pressure, compared to those offspring from mothers on the control diet [24]. Furthermore, upon examination of the DNA methylation status of 1400 CpG sites (mainly gene promoter-associated CpG islands dispersed throughout the genome) in fetal liver at gestational day 90, the methylation status of 4% of 1400 CpG islands was altered in offspring from the maternal folate-, vitamin B_{12}-, and methionine-deficient group. Interestingly, the vast majority (88%) of these loci were hypomethylated relative to controls, and most of the altered loci were specific to males.

A protein-restricted diet consumed throughout pregnancy has been shown to alter gene-specific DNA methylation, and maternal folate supplementation appears to reverse some of these effects in the offspring. For example, Lillycrop *et al*. examined the effect of a protein-restricted diet with or without folic acid supplementation from the time of conception

until gestational day 21 on the DNA methylation status of several genes in juvenile rat offspring [31]. A maternal diet restricted in protein led to a significant decrease in the methylation status of peroxisome proliferator–activated receptor (*Ppar*)-*α* and glucocorticoid receptor (*GR)* promoters in the liver of offspring, while the maternal protein-restricted diet with folic acid prevented this hypomethylation. This same group investigated the effect of a maternal protein-restricted diet on the methylation of specific CpG dinucleotides within the *Pparα* promoter in the liver of juvenile offspring [236]. The mean hepatic *Pparα* promoter methylation in offspring of the maternal protein-restricted diet was 26% lower than controls as a result of a reduction in methylation at CpG dinucleotides 2, 3, 4, and 16, while a maternal protein-restricted diet supplemented with folic acid prevented hypomethylation of these CpG dinucleotides in the offspring, and in addition, folic acid supplementation increased methylation at CpG dinucleotides 5 and 8, despite the fact there were no differences observed between these CpGs in the protein-restricted and control groups.

Gong *et al*. have also recently examined the effect of a maternal diet containing 180g/kg casein or 90 g/kg casein with either 1 mg/kg or 3 mg/kg folic acid on DNA methylation in the offspring [237]. Interestingly, a significant increase in DNA methylation in the imprinting control region (ICR) of the *Igf2/H19* locus was observed following a maternal low protein diet, while the protein-restricted diet supplemented with folic acid prevented this hypermethylation in offspring. Furthermore, the DMR2 did not differ in methylation patterns in offspring from any of the diet groups.

In another recent study, female rats were fed a diet containing either 9 or 18% casein by weight with either 1 or 5 mg/kg diet of folic acid throughout pregnancy until gestational day 20 [32]. Interestingly, fetal plasma homocysteine concentrations and fetal liver genomic

DNA methylation did not differ between any of the diet groups at gestational day 20. Furthermore, hepatic genomic DNA methylation was not altered in offspring of female rats fed a folate deficient diet for 2 weeks prior to mating and throughout pregnancy until gestational day 21 [168].

In contrast, alterations in genomic DNA methylation were observed in the *Folbp1* knockout mouse strain [238]. Finnell *et al*. examined the effect of maternal folate supplementation on genomic DNA methylation in *Folbp1* embryos of all possible genotypes. Female *Folbp1*$^{+/-}$ mice received a 25 mg/kg daily oral gavage of folic acid for two weeks prior to the first attempted mating and throughout pregnancy until gestational day 15.5. A decrease in hepatic genomic DNA methylation was observed in *Folbp1*$^{+/-}$ and *Folbp*$^{-/-}$ fetuses following folic acid supplementation compared to fetuses from unsupplemented controls; however, only the decrease in *Folbp*$^{-/-}$ fetuses was statistically significant. In the brain, a significant decrease in genomic DNA methylation was also observed in *Folbp1*$^{+/+}$ and *Folbp1*$^{+/-}$ fetuses.

Few human studies have examined the effect of maternal folate status on DNA methylation in the offspring. Recently, a preliminary prospective study conducted in the United Kingdom observed an inverse correlation between cord plasma homocysteine concentrations and LINE-1 methylation in cord lymphocyte samples from offspring of 24 women [239]. Furthermore, an association between LINE-1 methylation and birth weight centile was identified. Cord serum folate, however, was not associated with LINE-1 methylation.

In addition, a recent cross-sectional study in the Netherlands examined the effect of periconceptional folic acid use on DNA methylation of the *IGF2* DMR in offspring [232].

Whole blood samples and questionnaire data on periconceptional exposures were obtained at 17 months following delivery. In children of mothers who consumed periconceptional folic acid supplements (400 μg/day), methylation of the *IGF2* DMR in offspring whole blood was 4.5% higher compared to offspring who were not exposed to folic acid. The observed increase in DNA methylation associated with folic acid exposure is very similar to the 5.2% reduction in *IGF2* methylation following periconceptional exposure to famine during the Dutch Famine at the end of World War II [231]. Furthermore, the concentration of maternal SAM was also associated with greater *IGF2* DMR methylation in the child, and an inverse independent association was observed between *IGF2* DMR methylation and birth weight [232].

Table 2.13 Effects of maternal folate status on DNA methylation in the offspring

Study (Reference)	Species (Strain)	Maternal Diet with Folate	Duration of Maternal Diet	Endpoint	Organ/Tissue	DNA Methylation in Offspring	Outcome in Offspring
Cooney *et al*. 2002 [234]	A^{vy} mouse	Supplementation with folic acid, choline, and betaine, and vitamin B_{12}	2 weeks before until 1 week after pregnancy		Liver and kidney	A^{vy} proximal LTR	Increase and shift towards psuedoagouti phenotype
Waterland *et al*. 2003 [21]	A^{vy} mouse	Supplementation with folic acid, choline, and betaine, and vitamin B_{12}	2 weeks prior to mating, and throughout pregnancy and lactation	PND21 and PND100	Tail, liver, kidney, and brain	7 CpG sites in A^{vy} PS1A	Increase at each site
Dolinoy *et al*. 2007 [235]	A^{vy} mouse	BPA alone or BPA with vitamin B_{12}, betaine, choline, and 4.3 mg folic acid/kg	2 weeks prior to mating, and throughout pregnancy and lactation	PND22	Tail, brain, liver, and kidney	9 CpG sites in cryptic promoter of A^{vy} IAP	12% decrease (p=0.004) with BPA alone and methyl group supplementation reversed hypomethylation
Waterland *et al*. 2006 [22]	$Axin^{Fu}$ mouse	Supplementation with folic acid, choline, and betaine, and vitamin B_{12}	2 weeks prior to mating, and throughout pregnancy and lactation	PND21	Tail, kidney, liver, and brain	6 CpG sites spanning 3' IAP-genomic junction at $Axin^{Fu}$	Increase in tail No effect in kidney, liver, and brain
Sinclair *et al*. 2007 [24]	Sheep	Folate, vitamin B_{12}, and methionine deficient	8 weeks before until 6 days after conception	GD90	Liver	1400 CpG sites	4% of CpG sites had altered status ($p<0.001$)
Lillycrop *et al*. 2005 [31]	Rat (Wistar)	Protein restriction with 1 or 5 mg folic acid/kg	Pregnancy until GD21	PND34	Liver	*Pparα*, *GR* and *Pparγ*	21% decrease in *Pparα* ($p<0.001$) and 23% decrease in *GR* ($p<0.05$) with protein restriction, while folic acid supplementation reversed hypomethylation No effect on *Pparγ*
Lillycrop *et al*. 2008 [236]	Rat (Wistar)	Protein restriction with 1 or 5 mg folic acid/kg	Pregnancy	PND34	Liver	*Pparα*	40%, 43%, 33%, and 16% decrease ($p<0.05$) at CpGs 2, 3, 4, and 16, respectively with protein restriction,

							while folic acid supplementation reversed hypomethylation 47% increase at CpG 5 and 63% increase at CpG 8 with folic acid supplementation ($p<0.05$)
Gong *et al*. 2010 [237]	Rat (Sprague -Dawley)	Adequate protein or protein restriction with 1 or 3 mg folic acid/kg	Pregnancy	PND0	Liver	ICR of *Igf2*/*H19*	Increase with protein restriction, while folic acid supplementation reversed hypermethylation
						DMR2	No effect
Engeham *et al*. 2010 [32]	Rat (Wistar)	Adequate protein or protein restriction with 1 or 5 mg folic acid/kg	Pregnancy	GD21	Liver	Genomic	No effect
Maloney *et al*. 2007 [168]	Rat (Rowett Hooded)	Folate deficient	2 weeks before pregnancy until GD21	GD21	Liver	Genomic	No effect
Finnell *et al*. 2002 [238]	*Folbp1* Mouse	25 mg/kg/day folinic acid by gavage	2 weeks prior to mating and throughout pregnancy	GD15.5	Liver	Genomic	~ 4-fold decrease in $Folbp1^{+/-}$ ($p<0.05$)
					Brain		~ 2-fold decrease in $Folbp1^{+/+}$ and $Folbp1^{+/-}$($p<0.05$)
Fryer *et al*. 2009 [239]	Human	400 µg/day folic acid	Pregnancy	Term	Cord blood	Genomic	r=0.364 (p=0.08)
		Cord serum folate 15.8 µmol/L (mean)					r=0.209 (p>0.05)
		Cord tHcy 10.8 µmol/L (mean)					r=-0.688 (p=0.001)
Steegers-Theunissen *et al*. 2009 [232]	Human	400 µg/day folic acid	Periconceptional	17 months following delivery	Blood	*IGF2*	4.5% higher (p=0.014)

*A^{vy}=viable yellow agouti mouse model; LTR=long terminal repeat; PND=postnatal day; CpG= cytosine-guanine dinucleotide sequence; PS1A=pseudoexon 1A; BPA= bisphenol A; IAP=intra-cisternal A particle; GD=gestational day; *Ppar*=peroxisome proliferator-activated receptor; *GR*=glucocorticoid receptor; ICR=imprinting *control* region; *Igf2*=insulin-like growth factor 2; DMR=differentially methylated region; *Folbp1* =folate receptor 1; tHcy=homocysteine

Several animal studies in our laboratory have also shown that supplementing the maternal diet with folic acid throughout pregnancy and lactation can alter genomic and gene-specific DNA methylation in rat offspring (**Table 2.14**). In one study, maternal folic acid supplementation during pregnancy and lactation (5 mg folic acid/kg diet) was shown to increase global DNA methylation in the colonic mucosa in offspring at 3 weeks of age, while maternal folic acid supplementation during pregnancy and lactation had no effect on colonic global DNA methylation in offspring at 14 weeks of age [240]. However, the post-weaning diet of the offspring did alter genomic DNA methylation, as pups consuming a diet supplemented with folic acid (5 mg folic acid/kg diet) from weaning until 14 weeks of age demonstrated a significant 3-7% decrease in colonic genomic DNA methylation compared those pups that consumed the control diet. Interestingly, in the liver at both 3 and 14 weeks of age, genomic DNA hypomethylation was observed in offspring from the maternal folic acid supplemented group compared to those from the maternal control group. Furthermore, upon examination of gene-specific methylation in the offspring liver, maternal folic acid supplemention was shown to decrease *Pparγ*, *ER*-, *p53*-, and *Apc*-specific methylation, while at 14 weeks of age, maternal and pup folic acid supplementation (5 mg folic acid/kg diet) from 3 to 14 weeks increased methylation of *Pparγ*, *p53*, and *p16* and pup folic acid supplementation (5 mg folic acid/kg diet) from 3 to 14 weeks increased methylation of *ER* and *Apc*, in the offspring liver [241].

In another study, female rats were placed on either a control (2 mg folic acid/kg diet) or folic acid supplemented diet (5 mg folic acid/kg diet) 3 weeks prior to mating and during pregnancy and lactation [138]. At weaning, all female pups were randomized to either the control or supplemented diet and mammary tumors were induced with DMBA at 7 weeks of

age. Maternal folic acid supplementation was shown to induce a significant degree of global DNA hypomethylation in non-neoplastic mammary glands of the offspring.

Table 2.14 Animal studies from our laboratory examining the effect of maternal folic acid supplementation on DNA methylation in the offspring

Study (Reference)	Species (Strain)	Maternal Diet	Duration of Maternal Diet	Endpoint	Organ/ Tissue	DNA Methylation	Outcome in Offspring
Sie *et al*. [240] and Sie *et al*. 2010 [241]	Rat (Sprague-Dawley	Supplementation with 5 mg folic acid/kg diet	Pregnancy and lactation	3 weeks	Colonic mucosal tissue	Genomic	Increase (p=0.007)
					Liver	Genomic	Decrease (p<0.001)
						Gene-specific *Pparγ* (promoter) *p16* (promoter) *ER* (promoter) *p53* (exons 6 & 7) *Apc* (exon 15)	Decrease: *Pparγ*, *ER, p53*, and *Apc* (p<0.05)
				14 weeks	Colonic mucosal tissue	Genomic	Pup folic acid supplementation (5 mg folic acid/kg) from 3 to 14 weeks decreased methylation (p<0.05)
					Liver	Genomic	Decreased methylation in pups on supplemented diet (5 mg folic acid/kg diet from 3 to 14 weeks) (p=0.037)
						Gene-specific *Pparγ* (promoter) *p16* (promoter) *ER* (promoter) *p53* (exons 6 & 7) *Apc* (exon 15)	Maternal and pup folic acid supplementation (5 mg folic acid/kg diet from 3 to 14 weeks) independently increased methylation of *Pparγ*, *p53*, and *p16* (p<0.05) Pup folic acid supplementation (5 mg folic acid/kg diet) from 3 to 14 weeks increased methylation of *ER* and *Apc* (p<0.05)
Ly *et al*. 2010 [138]	Rat (Sprague-Dawley)	Supplementation with 5 mg folic acid/kg diet	Pregnancy and lactation	7 weeks	Non-neoplastic mammary gland	Genomic	Maternal folic acid supplementation decreased methylation in pups placed on control (2 mg folic acid/kg diet) from 3 to 7 weeks (p=0.03) Pup folic acid supplementation (5 mg folic acid/kg diet) from 3 to 7 weeks

							had no effect
				21 weeks			Maternal folic acid supplementation decreased methylation in pups placed on control (2 mg folic acid/kg diet) from 3 to 21 weeks ($p<0.001$) Maternal folic acid supplementation decreased methylation in pups placed on supplemented (5 mg folic acid/kg diet) from 3 to 21 weeks

*****_Ppar_=peroxisome proliferator-activated receptor*; p16*=cyclin-dependent kinase inhibitor 2A*; ER*=estrogen receptor; *p53*=tumor protein 53*; Apc*=adenomatous polyposis coli

2.2.5.2 Effects of Maternal Folate Status on Gene Expression in the Offspring

2.2.5.2.1 Effects of Folate Status on Gene Expression: Evidence from Animal Studies

Several of the previously mentioned animal studies investigating the effect of folate deficiency or supplementation on DNA methylation, as well as numerous additional studies, have examined the effect of folate status on gene expression **(Table 2.15)**. Sohn *et al*. observed that severe folate deficiency progressively decreased, while folate supplementation increased, steady-state levels of *p53* transcript in the colon over 5 weeks, although steady-state levels of *p53* mRNA did not correlate with genomic or *p53* methylation within the promoter region and exons 6-7 [25]. Liu *et al*. observed a decrease in colonic *p53* mRNA levels following isolated folate depletion for 10 weeks; however, this decrease only became statistically significant when mice consumed a diet deficient in both folate and vitamin B_{12}, or a diet deplete in multiple vitamins [178]. Furthermore, isolated folate deficiency induced a modest decrease in colonic MDM2 p53 binding protein homolog (*Mdm2)* expression; however, similar to *p53* gene expression, this was only statistically significant following combined vitamin depletion. In addition, folate depletion had no effect on *p21* expression. In another study, a 40% decrease in colonic *Apc* gene expression following folate depletion was observed; however, this decrease only became statistically significant when several vitamins were depleted [172]. A moderate degree of folate deficiency in pregnant female rats modulated the expression of several genes in the liver, including a 25% reduction in hepatic acetyl CoA carboxylase-1 (*Acaca*) mRNA levels and a 50% reduction in sterol regulatory element binding transcription factor 1 (*Srebp-1c*) mRNA levels [242].

Crott *et al*. examined the effects of folate status on gene expression in young and old rat colon [26]. In young rats fed a folate-deplete (0 mg/kg) diet, several immune-related genes were upregulated, while several adhesion molecules genes were downregulated compared to young rats fed a folate-supplemented (8 mg/kg) diet. Furthermore, young rats appeared to exhibit a more vigorous response to folate depletion, as 136 genes were altered in the young, while only 62 genes were altered in old rats following folate depletion, and genes differentially expressed differed greatly between both groups.

Table 2.15 Effects of folate deficiency and supplementation on gene expression in animal studies

Study (Reference)	Species (Strain)	Folate Level	Age Diet Began	Duration of Diet	Organ/Tissue	Gene Expression	Effect
Sohn *et al*. 2003 [25]	Rat (Sprague-Dawley)	0, 2, or 8 mg folic acid/kg diet	3 weeks	5 weeks	Colon	*p53*	Progressive decrease with deficiency and progressive increase with supplementation over 5 weeks ($p<0.05$, linear trend)
Liu *et al*. 2008 [178]	Mouse (C57BL/6)	Folate deficiency	4 months	10 weeks	Colon	*p53*	Modest decrease, only statistically significant when combined with vitamin B_{12} depletion or multiple vitamin depletion ($p<0.05$)
						Mdm2	Modest decrease, only statistically significant with multiple vitamin depletion
						p21	No effect
Liu *et al*. 2007 [172]	Mouse (C57BL/6)	0 or 2 mg folic acid/kg diet	4 months	10 weeks	Colon	*Apc*	No effect
McNeil *et al*. 2008 [242]	Rat (Rowett Hooded)	Folate deficiency	8-10 weeks	2 weeks prior to pregnancy until GD21	Liver	Genes involved in synthesis of fatty acids, fatty oxidation and transcriptional regulators that control them	Decrease in *Srebp-1c* and *Acaca* ($p<0.05$)
Crott *et al*. 2004 [26]	Rat (Sprague-Dawley)	0 or 8 mg folic acid/kg diet	3 weeks	20 weeks	Colon	Global gene expression analysis	136 genes altered: increase in immune-related genes, decrease in adhesion molecules (≥1.5 fold-change, $p<0.05$)
			12 months				62 genes altered (≥1.5 fold-change, $p<0.05$)

**p53*=tumor protein 53; *Mdm2*= Mdm2 p53 binding protein homolog; *p21*= cyclin-dependent kinase inhibitor 1A; *Apc*= adenomatous polyposis coli; GD=gestational day; *Acaca*= acetyl CoA carboxylase-1; *Srebp-1c*=sterol regulatory element-binding protein-1c

2.2.5.2.2 Effects of Folate Status on Gene Expression: Evidence from In Vitro Studies

Several *in vitro* studies have also investigated the effect of folate deficiency or supplementation on gene expression (**Table 2.16**). Jhaveri *et al*. examined the effect of folate deficiency on global gene expression in human nasopharyngeal carcinoma KB cells, and found that the expression of only 8 genes were affected by folate deficiency, and none of these were folate-related genes [27]. In contrast, Courtemanche *et al*. found that folate deficiency in human lymphocytes resulted in the down-regulation of 14 genes (many involved in folate metabolism), and the up-regulation of 24 genes (many involved in DNA repair and mitochondrial genes) [243].

In a recent study solely examining the expression of genes involved in folate metabolism in the colon cancer cell lines Caco-2 and HCT116, folate deficiency was shown to alter the expression of many genes involved in folate metabolism; however, the effect of folate deficiency appeared to be cell-specific [28]. In HCT116 cells, folate deficiency was shown to induce an adaptive regulation favouring an increase in folate uptake (upregulation of *FR-α*), the provision of metabolically more effective substrates for folate-dependent enzymes (upregulation of *FPGS*), and reduced folate hydrolysis (downregulation of *GGF*) and efflux (downregulation of *RFC*). In addition, folate deficiency was shown to shuttle the flux of one-carbon units to the methionine cycle (upregulation of *MTHFR*) to protect methylation reactions and therefore suppress DNA methylation (downregulation of *SHMT*, *TS*, and *DHFR*) in HCT116 cells. In contrast, certain adaptive responses following folate depletion were not as apparent in Caco2 cells, and the direction of the change was counterintuitive with certain genes. Furthermore, the metabolic priority following folate

deficiency in Caco2 cells was to shuttle available folate pools to the nucleotide biosynthesis pathway at the expense of the methionine cycle.

Additionally, results from an apoptosis and cancer pathway-specific mini-microarray analysis also suggest that the effect of folate deficiency on gene expression is highly cell-specific, as a low concordance rate of differentially expressed genes was observed between HCT116 and Caco-2 cell lines [244]. The expression of 7 most notably and consistently affected genes were confirmed by real time RT-PCR, and the response to folate deficiency was not uniformly consistent across four cell lines used (HCT116, Caco-2, HT29, and LS513). However, in 5 of the 7 genes examined, the change in gene expression was in the same direction in 3 of the 4 cell lines used.

In another study using 3 cell lines derived from the human colon (HCEC, NCM356, and NCM460), decreasing media folate resulted in an increase in expression of *FR*, *p53*, *p21*, *p16*, and β-catenin (*CTNNB1*) in all three cell lines, an increase in *APC* in NCM356 cells, and a decrease in expression of E-cadherin (*CDH1*) and SMAD family member 4 (*SMAD-4*) in NCM356 cells [186]. Furthermore, the induction of *p53* exons 5-8 breaks strand breaks from folate depletion did not appear to impair *p53* expression, as an approximate 2-fold increase in *p53* gene expression and a significant increase in the abundance of its protein were observed.

A recent study has also examined the effect of HT29 human colon cancer cells exposed to a chronic supplemental (100 ng/mL) level or a normal (10 ng/mL) level of folic acid on gene expression [245]. A relatively small cDNA microarray analysis representing 2200 genes revealed that the expression was significantly altered in only a small number of genes, none of which were related to folate metabolism.

Table 2.16 Effects of folate deficiency and supplementation on gene expression in *in vitro* systems

Study (Reference)	Cell Type	Folate Levels	Duration	Gene Expression	Effect
Jhaveri *et al*. 2001 [27]	Human nasopharyngeal epidermoid carcinoma KB cells	Folate deplete (2-10 nmol/L) vs. folate replete (2 µmol/L)	Unknown	Microarray analysis	3 genes upregulated and 5 genes downregulated (≥2-fold, $p<0.05$)
Courtemanche *et al*. 2004 [243]	Normal human T lymphocytes	Complete folate deficiency (0 nmol/L) or physiological folate deficiency (12 nmol/L) vs. folate-sufficient control cells (3000 nmol/L)	10 days	Microarray analysis	24 genes upregulated (many involved in DNA repair and mitochondrial genes) 14 genes downregulated (many involved in folate metabolism)
Hayashi *et al*. 2007 [28]	Human colon adenocarcinoma call lines (HCT116 and Caco-2)	Folate deficient vs. 2.3 µmol/L folic acid (control)	20 days	Genes involved in intracellular folate metabolism and 1-carbon transfer reactions	Many genes differentially expressed in both cell types, and cell-specific differences
Novakovic *et al*. 2006 [244]	Two human colon adenocarcinoma cell lines (HCT116 and Caco-2)	Folic acid deficient vs. control (2.3 µmol/L folic acid)	20 days	Apoptosis and cancer pathway-specific mini-microarray	Apoptosis genes: 2 upregulated and 3 downregulated in HCT116, 9 upregulated and 3 downregulated in Caco-2 cells Cancer genes: 11 upregulated and 2 downregulated in HCT116, 14 upregulated and 9 downregulated in Caco-2 cells 1 apoptosis and 6 cancer genes differentially expressed in both cell lines
	Four human colon adenocarcinoma cell lines (HCT116, Caco-2, HT29, and LS513)			Confirmation of 7 affected genes from microarray analysis with RT-PCR analysis	Direction and magnitude of change in expression not uniformly consistent across all four cell lines
Crott *et al*. 2008 [186]	Three colonic epithelial cell lines (HCEC, NCM460, and NCM356)	25, 50, 75, and 150 nmol/L of folic acid	30-32 days	Genes involved in cell-cycle checkpoints, intracellular signalling, folate uptake, and cell adhesion and migration	Increase in *FR*, *p53*, *p21*, *p16*, and *CTNNB1* expression for all cell lines with decreasing folate Decrease in *CDH1* and *SMAD-4* with decreasing folate in NCM356 cells

					Increase in APC with decreasing folate in NCM356 cells
Pellis *et al*. 2008 [245]	Human colon cancer cell line HT29	Normal folate (10 ng/mL folic acid) vs. supplemental folate (100 ng/mL folic acid)	3 weeks	Microarray analysis	10 genes upregulated and 6 genes downregulated with supplementation; genes involved in cell turnover and Fe metabolism

**FR*=folate receptor; *p53*=tumor protein; *p21*=cyclin-dependent kinase inhibitor 1A; *p16*=cyclin-dependent kinase inhibitor 2A; *CTNNB1*=β-catenin*; CDH1*=E-cadherin; *SMAD-4*=SMAD family member 4; *APC*= adenomatous polyposis coli

2.2.5.2.3 Effects of Folate Status on Gene Expression: Evidence from Human Studies

To date, several interventional and cross-sectional studies have investigated the effect of folate status on gene expression in humans (**Table 2.17**). In a study involving men with hyperhomocysteinaemia and uraemia undergoing standard haemodialysis treatment, supplementation with folate was shown to reduce hyperhomocysteinaemia and correct patterns of altered gene expression [29]. Seven patients heterozygous for *H19 Rsa*I restriction fragment length polymorphism were treated with the folate washout procedure for 2 months, followed by folate treatment (15 mg/day of methylTHF) for 8 weeks. Following the folate washout period, an increase in plasma homocysteine was observed. Following folate treatment, plasma homocysteine concentrations decreased in all patients and in general, reached concentrations lower than those observed prior to washout conditions. Furthermore, the pattern of allelic expression for the *H19* gene in peripheral mononuclear cells was examined, and a shift from monoallelic to biallelic expression was observed when plasma total homocysteine concentration was between 39 μmol/L and 62 μmol/L. Interestingly, in several patients with total homocysteine concentrations >62 μmol/L, a shift back to monoallelic expression of *H19* after following folate treatment was observed. In patients who had low total homocysteine concentrations <39 μmol/L demonstrating monoallelic expression after folate washout also had stable *H19* monoallelic expression after folate administration. Furthermore, expression of *IGF2* was negligible in patients with biallelic expression of *H19*, but was shown to significantly increase following folate treatment.

Using whole genome microarray analysis, a recent study investigated the gene expression profiles of endothelial progenitor cells from type 1 diabetes patients prior to and following a 4-week period of folic acid supplementation and in healthy controls (5 mg/day of folic acid) [246]. At baseline, diabetic patients demonstrated 1591 genes with altered expression compared to age- and gender-matched healthy subjects serving as controls. These altered genes were mainly involved in cell communication, development, localization, cell proliferation and cell adhesion. Interestingly, following folic acid supplementation, endothelial progenitor cell gene expression profiles from diabetic patients were similar to those from healthy controls, as the expression of 513 of the 1591 genes were normalized, and many of these genes were involved in development, cell adhesion and G protein signalling.

In addition, a recent study examined the relationship between the expression of the duodenal human *PCFT* with fasting RBC and serum folate concentrations in subjects undergoing diagnostic esophagogastroduodenoscopy [54]. The expression of *PCFT* mRNA varied 25-fold between subjects; however, a correlation between RBC or serum folate and *PCFT* expression was not observed.

In another study, the expression levels of several folate-related genes (*RFC*, *FPGS*, *GGH*, and *TS*) were examined in normal-appearing mucosa and tumor tissue of patients with CRC [247]. In mucosa, the total folate concentration correlated with gene expression levels of *RFC* and *FPGS*, while no correlation was observed between total folates and *TS* of *GGH* gene expression levels.

Table 2.17 Effects of folate status on gene expression in human studies

Study (Reference)	Subjects	Design	Folate Level	Gene Expression	Outcome
Ingrosso *et al*. 2003 [29]	Uremic patients with hyperhomocysteinemia undergoing haemodialysis treatment	Interventional	15 mg/day 5-methylTHF for 8 weeks	Lymphocytes *H19* and *IGF2*	Shift back to monoallelic expression of *H19* in patients with tHcy >62 µmol/L Increase in *IGF2* in patients with biallelic expression
van Oostrom *et al*. 2009 [246]	Patients with diabetes mellitus type 1	Interventional	5 mg/day folic acid for 4 weeks	Endothelial progenitor cells Microarray analysis	1591 genes altered compared to healthy controls, 513 of these were normalized with folic acid treatment
Urquhart *et al*. 2010 [54]	Subjects undergoing diagnostic esophagogastroduodenoscopy or colonscopy	Cross-sectional	Serum 634-2500 nmol/L (range)	Duodenum *PCFT*	No associations
Odin *et al*. 2003 [247]	Patients with colorectal cancer	Cross-sectional	Normal mucosa 3.0 nmol/g (mean) in patients with high FPGS expression 1.8 nmol/g (mean) in patients with low FPGS expression	Normal mucosa *RFC1 FPGS*, *GGH*, and *TS*	*RFC*: r=0.3 (p=0.05) *FPGS*: r=0.3 (p=0.05)

*THF=tetrahydrofolate; *IGF2*=insulin-like growth factor 2; tHcy=homocysteine; *PCFT*=proton-coupled folate transporter; *RFC*=reduced folate carrier; *FPGS*=folylpolyglutamate synthase; GGH=γ-glutamyl hydrolase; *TS*=thymidylate synthase

2.2.5.2.4 Effects of Maternal Nutrition (Excluding Folate) on Gene Expression in the Offspring: Evidence from Animal Studies

Exposure to a wide array of environmental factors, including cocaine, nicotine, and caffeine, during the earliest periods of life have been shown to alter the expression of various genes in the offspring [221, 248, 249]. However, the main focus of ***Chapter 2.2.5.2.4*** will be the influence of maternal nutrition (excluding diets containing folate) on gene expression in the offspring.

Numerous animal studies have examined the effect of maternal nutrition on gene expression in the offspring (**Table 2.18**). Several studies previously discussed that observed alterations in both genomic and gene-specific DNA methylation have also found significant changes in the expression of a variety of genes [31, 227]. Vucetic *et al*. found that maternal consumption of a high fat diet during pregnancy and lactation resulted in changes in dopamine and opiod gene expression in parallel with an increased drive for the consumption of palatable foods, which may drive the development of obesity [227].

In addition to these studies, a maternal diet high in fat consumed throughout pregnancy and lactation has been shown to increase mRNA levels of several genes including those regulating fetal growth, such as *Igf2*, in the liver of adult mice offspring. Interestingly, the body weight of offspring from mothers fed a high fat diet at weaning was significantly greater than offspring from mothers on the control diet, while no significant difference in body weights between the groups were observed in adult offspring. In addition, genes regulating fatty acid oxidation, including *Ppar*-α and carnitine palmitoyltransferase-1a (*Cpt1a*), were upregulated in the liver of adult mice offspring from the maternal high fat diet group [250]. In a previous study from this same group, a maternal high fat, high protein, and

low carbohydrate diet was shown to increase protein levels of PPARα and CPT-1A and reduce hepatic lipid levels in offspring [251].

Furthermore, Su *et al*. recently conducted a genome-wide gene expression analysis of mammary tissue in weanling rats exposed to soy protein isolate *in utero* and during lactation, and found that relative to offspring from dams who consumed the control diet, 18 genes were upregulated and 39 genes were downregulated [252]. Many of the differentially expressed genes were involved in *de novo* synthesis of fatty acids, fatty acid desaturation, cholesterol production, and glucocorticoid activation.

In another study, in the liver of adult male offspring from dams fed a protein-restricted diet during pregnancy, 311 genes were differentially expressed compared to offspring from control dams, including genes associated with ion transport, developmental process, and response to reactive oxygen species and steroid hormone response [30].

Table 2.18 Effects of maternal nutritional factors (excluding folate) on gene expression in the offspring in animal studies

Study (Reference)	Species (Strain)	Maternal Diet	Duration of Maternal Diet	Endpoint	Organ/ Tissue	Gene Expression	Effect
Vucetic *et al*. 2010 [227]	Mouse (C57BL/6)	High fat	3 months prior to mating, pregnancy, and lactation	PND126-168	Male brain (4 regions: VTA, PFC, NAc, hypothalamus)	*Th*, *Dat*, *Comt*, *D1*, *D2*, and *Darpp-32*	Increase (VTA, Nac, and PFC) and decrease (hypothalamus) in *Dat* ($p<0.05$) Increase in *Th* (hypothalamus) ($p<0.05$) Decrease in *D1*, *D2*, and *Darpp-32* (Nac and PEC) ($p<0.05$) No effect on *Comt*
Lillycrop *et al*. 2005 [31]	Rat (Wistar)	Protein restricted	Pregnancy	PND34	Liver	*GR, Pparα, Pparγ, Aox*	Increase in *Pparα*, *GR,* and *Aox* ($p<0.0001$)
Zhang *et al*. 2009 [250]	Mouse (C57BL/6)	High fat	4 weeks prior to conception, during pregnancy and lactation	PND105	Liver	*Pparα*, *Cpt1a* and *Igf2*	Increase in *Pparα* ($p<0.05$), *Cpt1a* ($p<0.01$), and *Igf2* ($p<0.01$)
Su *et al*. 2009 [252]	Rat (Sprague Dawley)	High in isoflavones	GD4, remainder of pregnancy and lactation	PND21	Mammary gland	Gene expression profile (microarray)	57 genes differentially expressed (associated with *de novo* synthesis of fatty acids, fatty acid desaturation, cholesterol production) (fold change ≥ 1.3; $p<0.05$)
Lillycrop *et al*. 2010 [30]	Rat (Wistar)	Protein restricted	Pregnancy	PND84	Liver	Gene expression profile (microarray)	311 genes differentially expressed (associated with ion transport, developmental process, and response to reactive oxygen species and steroid hormone response) (fold change ≥ 1.5; $p<0.05$)

*PND=postnatal day; VTA=ventral tegmental area; PFC=prefrontal cortex; NAc=nucleus accumbens, *Th*=tyrosine hydroxylase; *Dat*=dopamine reuptake transporter; *Comt*=catechol-*O*-methyltransferase; *D1*=dopamine receptor 1; *D2*=dopamine receptor 2; *Darpp-32*=dopamine- and cAMP-regulated phosphoprotein; *Gr*=glucocorticoid receptor; *Ppar*=peroxisomal proliferator–activated receptor; *Aox*=acyl-CoA oxidase; *Cpt1a*=carnitine palmitoyltransferase-1a; *Igf2*=insulin-like growth factor 2; GD=gestational day

2.2.5.2.5 Effects of Maternal Folate Status on Gene Expression in the Offspring

Several animal studies have investigated the effect of altered maternal folate status alone or in combination with a protein-restricted diet on gene expression in the offspring (**Table 2.19**). In several studies previously mentioned examining the effect of maternal folate status on DNA methylation in the offspring, changes in gene expression in the offspring have also been investigated [31, 32, 237]. In one study by Lillycrop *et al*., *GR* and *Pparα* promoters were hypomethylated in offspring liver from mothers fed a protein-restricted diet and a significant increase in mRNA expression of both genes was observed, while folic acid supplementation in addition to the maternal protein-restricted diet prevented this increase in expression [31]. In addition, a diet low in protein consumed during pregnancy was shown to increase ICR *Igf2/H19* DNA methylation and *Igf2* and *H19* mRNA expression in the liver of offspring, while the addition of folic acid to the protein-restricted diet prevented this increase [237]. Furthermore, significant increases in mRNA levels of *Dnmt1, Dnmt3a*, and *Mbd2* were observed in offspring liver following the maternal protein-restricted diet; however, folic acid supplementation prevented these increases. In the study by Engeham *et al*., the expression of *Dnmt1*, *Mthfr*, and *Mtr* mRNA were not altered in fetal liver from mothers fed 5 mg folic acid/kg diet, compared to offspring from mothers fed the control diet [32].

A maternal diet deficient in folate consumed for 2 weeks prior to pregnancy until gestational day 21 was shown to significantly increase *Cpt1a* mRNA levels and decrease mRNA levels of CD36 molecule (*CD36*) in the offspring [253]. Interestingly, maternal folate deficiency appeared to mainly affect lipid metabolism in the maternal system, while only compensatory changes in fatty acid oxidation in the fetal liver were observed. Furthermore,

folate deficiency combined with multiple methyl donor deficiency induced a more widespread change in the expression of genes related to fat metabolism.

In contrast to a candidate gene approach, several recent studies to date have used a genome-wide microarray analysis to investigate the effect of maternal folate status, in combination with other dietary components or environmental exposures on global gene expression patterns in the offspring. In one study, a genome-wide analysis was performed in adult offspring from pregnant rats that consumed a protein-restricted diet alone or in combination with supplemental levels of folic acid (5 mg/kg folic acid) [30]. Offspring from mothers fed a protein-restricted maternal diet group displayed altered gene expression, and maternal folic acid supplementation was shown to correct some, but not all, of these changes. In certain cases, genes that were not differently expressed from the maternal protein-restricted diet alone demonstrated significant changes following folic acid supplementation. Following gene ontology analysis, offspring from the maternal protein-restricted/folic acid supplementation group demonstrated alterations in genes involved in fatty acid and steroid metabolic process pathways, while these were not altered in offspring from the maternal protein-restricted group, suggesting that folic acid supplementation induces distinct changes in gene expression.

In another recent study, Caldwell *et al*. examined the effect of maternal folate deficiency and supplementation alone or with exposure to low doses of trichloroethylene (TCE) during pregnancy on transcriptional changes in the embryonic heart [254]. Maternal TCE exposure resulted in extensive alterations in the expression of genes involved in transport, ion channel, transcription, differentiation, cytoskeleton, cell cycle, and apoptosis. Furthermore, exogenous folate did not appear to offset the effects of TCE exposure.

Interestingly, the most pronounced changes in gene expression were from alterations in dietary folate alone, suggesting a drastic effect of folate on gene expression. In fetuses exposed to folate deficiency, more than 50 transcripts involved in the apoptosis and cytoskeleton pathways, and more than 70 transcripts involved in the differentiation pathway, were altered in the heart. In fetuses exposed to folate supplementation, more than 80 transcripts in the differentiation, more than 60 transcripts in the adhesion, apoptosis, and cell cycle, and more than 50 transcripts in the cytoskeleton pathways, were altered in the heart.

Table 2.19 Effects of maternal folate status on gene expression in the offspring

Study (Reference)	Species (Strain)	Maternal Folate Level	Duration of Maternal Diet	Endpoint	Offspring Organ/ Tissue	Gene Expression in Offspring	Outcome in Offspring
Lillycrop *et al*. 2005 [31]	Rat (Wistar)	Protein restriction with 1 or 5 mg folic acid/kg diet	Pregnancy until GD21	PND34	Liver	*Pparα*, *Gr, and Aox*	945% ($p<0.0001$), 300% ($p<0.001$), and 217% ($p<0.001$) increase with in *Pparα*, *Gr* and *Aox*, respectively, with protein restriction Folic acid supplementation prevented increases
Gong *et al*. 2010 [237]	Rat (Sprague-Dawley)	Adequate protein or protein restriction with 1 or 3 mg folic acid/kg diet	Pregnancy	PND0	Liver	*Igf2*, *H19*, *Dnmt1*, *Dnmt3a*, and *Mbd2*	Increase with protein restriction, Folic acid supplementation prevented increase
Engeham *et al*. 2010 [32]	Rat (Wistar)	Adequate protein or protein restriction with 1 or 5 mg folic acid/kg diet	Pregnancy	GD21	Liver	*Dnmt1*, *Mthfr*, *Mtr*	No effect
McNeil *et al*. 2009 [253]	Rat (Rowett hooded)	Folate deficiency	2 weeks prior to mating until GD21	GD21	Liver	Genes coding for enzymes involved in lipid metabolism	173% increase in *L-cpt-1* ($p<0.05$) and 40% decrease in *Cd36* ($p<0.05$)
Lillycrop *et al*. 2010 [30]	Rat (Wistar)	Protein restriction with 1 or 5 mg folic acid/kg diet	Pregnancy	PND84	Liver	Microarray analysis	Protein restriction altered 311 genes, while folic acid supplementation corrected for 120 genes Folic acid supplementation altered expression of genes involved in fatty acid and steroid metabolic process pathways, and two imprinted genes: *Ins1* and *Phlda2*
Caldwell *et al*. 2010 [254]	Mouse (129S1/SvlmJ)	0, 2, or 8 mg folic acid/kg diet	4 weeks before mating and	GD10	Heart	Microarray analysis	Folate deficiency induced changes in >50 transcripts involved in the

			throughout pregnancy until GD10 (folate)				apoptosis and cytoskeleton pathways, and > 70 transcripts involved in the differentiation pathway Folate supplementation induced changes in >80 transcripts in the differentiation, >60 transcripts in the adhesion, apoptosis, and cell cycle, and >50 transcripts in the cytoskeleton pathways

*GD=gestational day; PND=postnatal day; *Ppar*=peroxisome proliferator-activated receptor; *Gr*=glucocorticoid receptor; *Aox*=aldehyde oxidase; *Igf2*=insulin-like growth factor 2; *DNMT*=DNA methyltransferase; *Mbd2*=methyl-CpG binding domain protein 2; *Mthfr*=methylenetetrahydrofolate reductase; *Mtr*=methionine synthase; *L-cpt-1*=liver type carnitine palmitoyl transferase-1*; Cd36*=CD36 molecule

2.2.5.2.6 Summary of the Effects of Folate Status on DNA Methylation and Gene Expression

Evidence from *in vitro*, animal, and human studies suggests that the effects of both folate deficiency and supplementation on DNA methylation are highly complex and appear to depend on cell type, target organ, and stage of transformation. Furthermore, the effect of an altered folate status on DNA methylation appears to be gene and site-specific, and depend on the magnitude, duration, and timing of folate manipulations, on interactions with other methyl group donors and dietary factors, and on genetic variants in the folate metabolic and one-carbon transfer pathways.

In vitro, animal, and human studies also suggest that both folate deficiency and supplementation are capable of modulating the expression of numerous genes involved in a wide array of biological functions. Similar to DNA methylation, the effects of folate deficiency and supplementation on gene expression are highly complex, and appear to depend on numerous factors including cell type, stage of transformation, and the magnitude, duration, and timing of folate manipulations.

Therefore potential for folate to modulate DNA methylation and gene expression and thus modify the risk of chronic diseases in humans is worthy of further studies.

Chapter 3: RATIONALE, OBJECTIVES, AND HYPOTHESIS

Rationale: Mandatory folic acid fortification has been highly successful in achieving its primary objective – that is the reduction of NTDs in Canada and the United States [5-8]. In addition, implementation of the program has effectively improved folate status in North America, as a dramatic rise in blood folate measurements has been observed across all gender and age groups [9]. In women of childbearing age in particular, large increases in serum and RBC folate concentrations have been observed since the initiation of the program [10]. In addition to folic acid fortification, widespread consumption of multivitamins containing folic acid and current recommendations advising women of childbearing age to consume a prenatal folic acid supplement have likely contributed to the large increase in blood folate status in women of childbearing age [5-8]. Currently, it is recommended that women of childbearing age consume a minimum of 400 μg folic acid daily from food and/or supplements, and many prenatal multivitamins contain 1 mg of folic acid [13]. In addition, the SOGC and Motherisk have recently recommended that certain women of childbearing age, such as those with a history of poor adherence and specific health conditions, consume a daily supplement containing 5 mg of folic acid [12].

A high maternal folate intake throughout pregnancy and lactation not only exposes the mother to high levels of folate, but also exposes the developing embryo, fetus, and infant to elevated levels of this vitamin. At present, the effects of intrauterine and early postnatal exposure to high levels of folate on the health of the offspring are largely unknown. However, emerging evidence suggests that early life exposure to environmental stimuli, including dietary factors, may influence the risk of disease later in life – a process known as 'fetal programming' [14]. Recent human studies have suggested that increased exposure to

folate during the earliest stages of life may increase the risk of diseases including obesity, insulin resistance, and asthma in the offspring later in life [15-17]. In addition, animal studies have suggested that maternal folic acid supplementation may modulate the risk of cancer development in the offspring in a site-specific manner [132, 138].

DNA methylation, an epigenetic modification of gene expression, has been suggested as one possible mechanism by which dietary factors such as folate can induce fetal programming. Folate plays an essential role in the provision of SAM, the major methyl donor for numerous biological methylation reactions including that of DNA. Although not uniformly consistent, both folate deficiency and supplementation in the young, during adulthood, and in the elderly, have been shown to be able to modulate DNA methylation patterns [18, 19]. During embryogenesis, DNA methylation patterns are reprogrammed as the epigenome undergoes widespread demethylation resulting in the erasure of significant parts of the parental DNA methylation, followed by *de novo* methylation in which new DNA methylation patterns are established [20]. Thus, the earliest stages of life may be a time in which the epigenome is most sensitive to environmental influences, including high levels of folate and folic acid.

A high maternal folate status could alter the methylation of CpG sites located in the bulk of the genome including exons, noncoding regions, and repeat DNA sites. In addition, intrauterine and early postnatal exposure to high levels of folate could modulate the methylation status of CpG islands spanning the 5' end of human genes including the promoter region, untranslated region, and exon 1, which may lead to persistent changes in offspring gene expression and phenotype, and ultimately influence the subsequent risk of disease.

Numerous *in vitro*, animal, and human studies have demonstrated that both folate deficiency and supplementation in the young, during adulthood, and in the elderly are capable of modulating the expression of various genes [25-29]. Thus, maternal folic acid supplementation throughout pregnancy and lactation may also be capable of altering the expression of genes in the offspring. Several recent animal studies suggest that maternal folic acid supplementation in combination with a protein restricted diet can alter gene expression in the offspring [30-32]. Furthermore, a recent study also suggests that maternal folic acid supplementation of a moderate degree throughout the duration of pregnancy can alter the expression of numerous genes in the fetal heart [254].

Thus, mandatory folic acid fortification and periconceptional folic acid supplementation, originally intended to reduce the incidence of NTDs, may also be accompanied with other biological effects on the developing offspring.

To date, most animal studies examining the effect of maternal folate status on DNA methylation and gene expression patterns in the offspring have utilized other dietary components in combination with supplemental levels of folate [21, 24, 30, 31, 237]. In addition, animal studies have not investigated the effect of varying doses of maternal folic acid supplementation, including very high levels paralleling the recent SOGC and Motherisk recommendations in humans, on DNA methylation and gene expression in the offspring. Furthermore, to our knowledge, the effect of isolated maternal folic acid supplementation provided during both pregnancy and lactation on genome-wide gene expression patterns in a mouse model has not been investigated. Thus, the specific genes and biological pathways in the offspring affected by maternal folic acid supplementation throughout pregnancy and lactation remain predominantly unknown. In order to determine whether folic acid

supplementation during pregnancy and lactation may influence the long-term health of the offspring, it is important to evaluate the effect of maternal isolated folic acid supplementation, of varying doses, provided during the intrauterine and early postnatal period on DNA methylation and global gene expression patterns in the developing offspring.

Objectives: To determine whether increasing doses (2.5x, 4x, and 12.5x BDR) of folic acid supplementation provided *in utero* and during lactation would modulate i) global DNA methylation in the liver of juvenile and adult offspring, and ii) global gene expression patterns in the liver of juvenile offspring.

Hypothesis: Based on the essential role of folate in DNA methylation, we hypothesize that maternal folic acid supplementation throughout pregnancy and lactation will increase global DNA methylation in juvenile offspring. Although the offspring's exposure to folic acid supplementation will not continue following lactation, we hypothesize that these changes in global DNA methylation, resulting from maternal folic acid supplementation provided *in utero* and during lactation, will persist into adulthood. Furthermore, we hypothesize that maternal folic acid supplementation during the *in utero* and early postnatal periods will modulate the expression of numerous genes in juvenile offspring.

Chapter 4: THE EFFECTS OF MATERNAL FOLIC ACID SUPPLEMENTATION PROVIDED IN UTERO AND DURING LACTATION ON GENOMIC DNA METHYLATION IN THE OFFSPRING

4.1 Introduction

Despite the fact that DNA methylation patterns remain relatively fixed throughout the life course, these patterns have been shown to change during certain disease states [20, 147]. In cancer, for example, abnormal patterns of DNA methylation are often observed, as loss of DNA methylation in the CpG-depleted regions has been shown to occur where most CpG dinucleotides are normally methylated, and methylation of CpG islands in gene promoter regions occurs where most CpG dinucleotides are normally unmethylated [147].

In addition to certain disease states, the epigenome may also be particularly susceptible to dysregulation during different developmental stages, including gestation, neonatal development, puberty, and old age [20]. However, it has been suggested that the epigenome is most sensitive to environmental insults during embryogenesis, a period in which extensive reprogramming of the epigenome occurs. In fact, early life nutrition has been shown to influence the establishment of epigenetic marks in the developing offspring. For example, Waterland and Jirtle found that supplementing a mother's nutritionally adequate diet with extra methyl donors, including folic acid, can permanently affect the offspring's DNA methylation at epigenetically susceptible loci [21].

The major biochemical role of folate is to mediate the transfer of one-carbon moieties [2]. For this reason, folate plays a critical role in the synthesis of SAM, a major methyl donor in most biological methylation reactions, including that of DNA. Folate deficiency and supplementation in the young, during adulthood, and in the elderly, have been shown to

modify DNA methylation patterns [18, 19]. In addition, several preliminary animal studies from our laboratory suggest that intrauterine and/or early postnatal exposure to high levels of folate may also influence DNA methylation patterns in the developing rat offspring [132, 138].

At present, however, it is unknown whether maternal folic acid supplementation can modulate DNA methylation in mouse offspring in a dose-dependent manner, and whether any observed effects will persist into adulthood. Thus, the objective of the present study is to investigate the effect of maternal folic acid (5, 8, and 25 mg folic acid/kg diet) supplementation during pregnancy and lactation on DNA methylation in both juvenile and adult offspring. Due to the essential role of folate in DNA methylation, we hypothesize that maternal folic acid supplementation during pregnancy and lactation will increase DNA methylation in juvenile offspring. Although the offspring's exposure to supplemental levels of folic acid will cease following lactation, we hypothesize that these newly established patterns of DNA methylation will persist into adulthood.

4.2 Materials and Methods

4.2.1 Experimental Design

C57BL/6 mice (40 females and 20 males) were purchased at 4 weeks of age from Charles River Laboratories (St. Constant, Quebec, Canada), and were provided with free access to water and control (2 mg folic acid/kg diet) diet for 1 week prior to the initiation of the experimental diets.

At 5 weeks of age, the experimental diets commenced (**Figure 4.1**). At this time, female mice were randomly assigned to one of four diets: the control diet (2 mg folic acid/kg diet) or a folic acid supplemented diet (5, 8, or 25 mg folic acid/kg diet). A total of 10 females were assigned to each diet group. Breeding commenced 3 weeks after the initiation of the diets. Breeding pairs were organized in a male:female ratio of 1:2. Upon detection of pregnancy, females were separated from males, housed individually, and continued to consume the experimental diets throughout pregnancy. Litters born (0 weeks) remained with their mothers throughout the duration of lactation, and mothers remained on the experimental diets throughout this period.

At the time of weaning (3 weeks), all pups were separated from their mothers and were weighed. Pups were randomly assigned either to be sacrificed at 3 weeks or to consume the control diet from 3 weeks until adulthood (18 weeks), as follows:

- To the extent possible, given the number of pups of each sex born to each mother, 1 male pup and 1 female pup from each mother were selected at random for immediate sacrifice at 3 weeks
- Of the remaining pups, 1 male pup and 1 female pup from each mother were selected (randomly, if there was more than one remaining pup of either sex) to continue on the control diet until the final sacrifice in adulthood at 18 weeks
- If the mother had more than 2 pups of the same sex, the remaining pups were assigned to immediate sacrifice or to the control diet, in such a way as to end up with as close as possible to equal numbers of pups in the four groups (male/female x immediate sacrifice/control diet)

At 3 weeks of age, pups that were selected to remain on the control diet were placed into polycarbonate cages of 4 according to their sex. Body weight of the pups was recorded weekly. Mice were housed at 24±2 ºC at 50% humidity with a 12 hour light/dark cycle. For ethical reasons, wire bottom stainless steel cages were not used. Animal care and use were within the Canadian Council on Animal Care (1984) guidelines. The experimental protocol was approved by The Animal Care Committee at the University of Toronto.

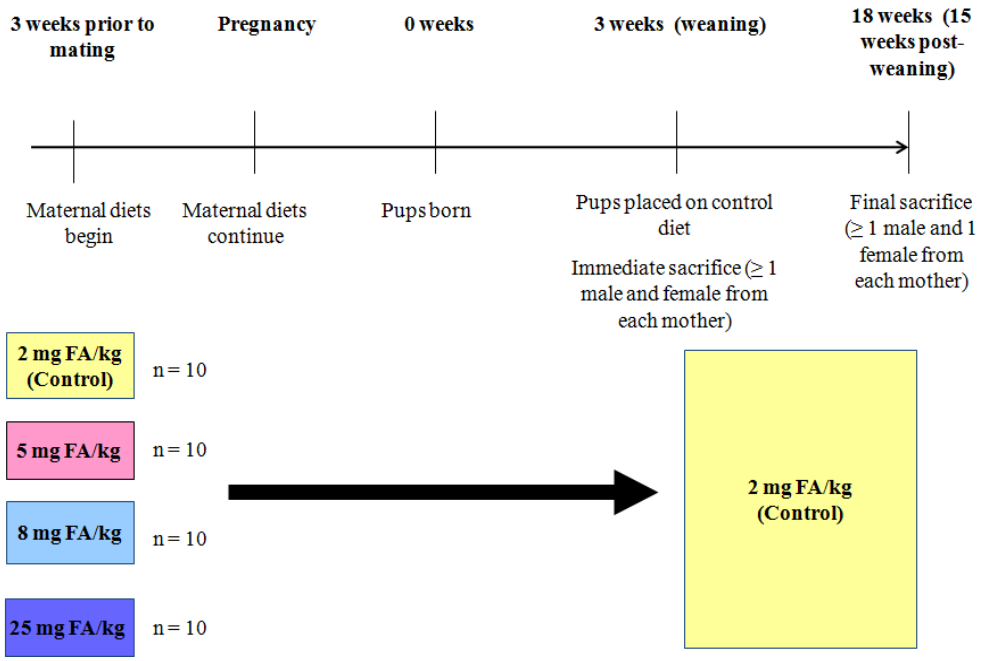

Figure 4.1 Experimental study design. FA=folic acid.

4.2.2 Experimental Diets

Mice were fed L-amino-acid defined diets in pellet form (Dyets, Bethlehem, PA). These diets have been shown to modulate folate levels in rodents [255], and have been used extensively in our laboratory in previous studies [19, 25, 131, 170]. Diets and water were provided *ad libitum* throughout the study.

Each of the diets utilized throughout the current study contain different levels of folic acid. The control diet contains 2 mg folic acid/kg diet and is generally accepted as the basal dietary requirement (BDR) for rodents. The control diet used in the present study represents the RDA in humans, set at 400 μg DFEs. This was determined when taking into consideration that the experimental diets contain approximately 4000 kcal/kg diet. In 2000 kcal, there is approximately 0.5-1 mg of folic acid. Due to the fact humans are estimated to consume approximately 2000 kcal per day, this amount of folic acid expressed relative to caloric content is very similar to the RDA for humans set at 400 μg/day DFEs. In North America, the average total folate intake postfortification is estimated to be approximately 400 μg/day in supplement nonusers and 800 μg/day for those taking multivitamins containing folic acid [256]. Therefore, for women of childbearing age in North America, it is likely that the average folate intake (400 μg/day for nonsupplemental users and 800 μg/day for those consuming multivitamins) consuming the recommended periconceptional folic acid supplements (400 μg-1000 μg) ranges from 800 μg/day to 1800 μg/day (2x-4.5x RDA). Thus, the supplemental diets containing 5 and 8 mg folic acid/kg diet were selected to represent the lower and higher total intake of this range (2.5x and 4x BDR, respectively). In addition, the 25 mg folic acid/kg diet (12.5x BDR) diet was selected to represent the recent recommendation by the SOGC and Motherisk, advising certain pregnant women, such as

those with poor compliance or specific health risks, to consume a daily supplement containing 5 mg of folic acid (12.5x RDA) during pregnancy and lactation.

All other ingredients were identical in each of the 4 diets. **Tables 4.1-4.3** list the exact composition of the diets. Diets were stored at 4ºC, and food in the cages was refilled every 3 days.

Table 4.1 Ingredient composition of experimental diets

Nutrient (g/kg)	Control Diet (2 mg Folic Acid)	Supplemented Diet (5 mg Folic Acid)	Supplemented Diet (8 mg Folic Acid)	Supplemented Diet (25 mg Folic Acid)
L-Alanine	3.5	3.5	3.5	3.5
L-Arginine (free base)	11.2	11.2	11.2	11.2
L-Asparaginine	6.0	6.0	6.0	6.0
L-Aspartic Acid	3.5	3.5	3.5	3.5
L-Cystine	3.5	3.5	3.5	3.5
L-Glutamic Acid	35.0	35.0	35.0	35.0
Glycine	23.3	23.3	23.3	23.3
L-Histidine (free base)	3.3	3.3	3.3	3.3
Isoleucine	8.2	8.2	8.2	8.2
L-Leucine	11.1	11.1	11.1	11.1
L-Lysine HCl	14.4	14.4	14.4	14.4
L-Methionine	8.2	8.2	8.2	8.2
L-Phenylalanine	11.6	11.6	11.6	11.6
L-Proline	3.5	3.5	3.5	3.5
L-Serine	3.5	3.5	3.5	3.5
L-Threonine	8.2	8.2	8.2	8.2
L-Tryptophan	1.74	1.74	1.74	1.74
L-Tyrosine	3.5	3.5	3.5	3.5
L-Valine	8.2	8.2	8.2	8.2
Total L-Amino Acid	**171.44**	**171.44**	**171.44**	**171.44**
Dextrin	407	408	407.4	404
Sucrose	193	193	193	193
Cellulose	50	50	50	50
Corn Oil (Stab. 0.015% BHT)	100	100	100	100
Mineral and Salt Mix 3210006	57.96	57.96	57.96	57.96
Vitamin Mix #317756	10	10	10	10
Choline Chloride	2	2	2	2
Sodium Bicarbonate	6.6	6.6	6.6	6.6
Folic Acid/Sucrose Premix (1 mg/g)	**2.0**	**5.0**	**8.0**	**25.0**

*L-Amino acid defined diets (Dyets, Bethlehem, PA)
Dextrin was made by partially hydrolyzing cornstarch using heat and a mineral acid (HCl, H_2SO_4)

Table 4.2 Tufts folate deficient mineral and salt mix used in amino acid defined diets

Minerals (g/kg diet) – Salt Mix #210006	
Calcium carbonate	14.6000
Calcium phosphate, dibasic	0.17000
Sodium chloride	12.37000
Potassium phosphate, dibasic	17.16000
Magnesium sulphate, anhydrous	2.45000
Maganese sulphate, monohydrate	0.18000
Ferric citrate	0.62000
Zinc carbonate	0.05400
Cupric carbonate	0.05400
Potassium iodide	0.00058
Sodium selenite	0.00058
Chromium potassium sulphate	0.01900
Sodium fluoride	0.00230
Molybdic acid, ammonium salt	0.00120
Sucrose	10.27534

Table 4.3 Tufts folate deficient vitamin mix used in amino acid defined diets

Vitamins (g/kg diet) - #317756 Folate Free	
Thiamin HCl	0.006
Riboflavin	0.006
Pyridoxine HCl	0.007
Nicotinic acid	0.030
Calcium pantothenate	0.016
Cyanocobalamin	0.00005
Vitamin A palmitate (500,000 IU/g)	0.008
Vitamin D3 (400,00 IU/g)	0.0025
Vitamin E acetate (500 IU/g)	0.100
Menadionine sodium bisulphate	0.00080
Biotin	0.00002
Sucrose	9.82363

4.2.3 Sample Collection

At 3 and 18 weeks of age, pups were sacrificed by carbon dioxide inhalation followed by cervical dislocation. Blood was obtained via cardiac puncture with the use of a pre-heparinized 23 gauge needle in a 1 mL sterile syringe, and was immediately placed on ice. Following the removal from the ice, blood samples were left at room temperature for 30 minutes, and centrifuged for 10 minutes at 2500 rpm at 4 ºC. Plasma was obtained (~100 µL and ~500 µL at 3 weeks and 18 weeks, respectively) and was aliquoted into Eppendorf tubes. For future plasma folate analysis, 2.5 µL of 1% ascorbic acid was added to 47.5 µL of plasma. The remainder of plasma was designated for homocysteine analysis. Samples were then stored at -80ºC.

At the time of sacrifice (3 weeks and 18 weeks), the liver was excised, immediately snap frozen in liquid nitrogen, and stored at -80 ºC. Other organs such as the colorectum were excised, processed, immediately frozen in liquid nitrogen, and then stored at -80 ºC for future studies.

4.2.4 Plasma and Liver Folate Concentrations

Folate concentrations were determined in both plasma and liver, using a standard microbiological *Lactobacillus rhamnosus* (formerly known as *L. casei*) microtiter plate technique that has been previously described [257]. *L. rhamnosus* is dependent on folate and cannot grow in a folate-free medium. Thus, the growth response is proportional to the amount of folate in the sample. The turbidity of the media, as a result of bacteria growth, can

then be measured by a spectrophotometer in order to determine the folate concentration in the sample.

Folic Acid Standard Preparation:

In 10 mL of double distilled water (ddH_20), 10 mg of folic acid was dissolved with 5 µL of 10N NaOH, resulting in a final concentration of 1 mg folic acid/mL. The pH was adjusted with HCl (pH 7-8), and the concentration was verified with the use of a spectrophotometer (282 nm). The solution was then diluted (2 ng/mL) in sterile filtered 0.1 M KPO_4 buffer (1.05 g KH_2PO_4, 0.4 g K_2HPO_4, 0.1 g sodium ascorbate, 100 mL ddH_20). The resultant folic acid standard was stored in aliquots at -80 ºC.

Lactobacillus Rhamnosus Stock Preparation:

In 200 mL of Lactobacillus MRS broth, 250 µL of *L. rhamnosus* stock (ATCC 7469 stock) was grown for 18 hours at 37 ºC. In an aseptic environment, the cells were centrifuged and the supernatant was decanted. The cell pellet was resuspended in 180 mL of Lactobacillus MRS broth, and 20 mL of 100% autoclaved cold glycerol was added. After mixing well, the solution was stored in aliquots at -80 ºC.

Chicken Pancreas Conjugase Preparation:

In 0.1 M KPO_4 buffer, chicken pancreas acetone powder (Difco 0459-12-2) was dissolved and incubated under a blanket of toluene for 6 hours at 37 ºC. The solution was centrifuged in glass Corex tubes at 10,000 x g for 15 minutes, the supernatant was collected

and an equal volume of tricalcium phosphate was added (BioRad Gel HTP was rehydrated: 1 part HTP to 6 parts 0.1 M KPO_4 buffer per 10 g HTP). The solution was stirred at 4 ºC for 30 minutes and centrifuged at 10,000 x g for 30 minutes at 4 ºC. The supernatant was collected, cooled to 4 ºC, and 95% ethanol (an equal volume) was then added and mixed. This was then left overnight at -20 ºC. The following day, the solution was centrifuged again at 10,000 x g for 30 minutes. The collected supernatant was resuspended in 50 mL of cold 1.0 M KPO_4 buffer, mixed with 10 g of Dowex-1 (BioRadAG1-X8), and stirred for 1 hour at 4 ºC. The mixture was then filtered through Whatman #1 filter paper at 4 ºC, and stored in aliquots at -80 ºC.

Liver Tissue Folate Extraction:

Liver tissue was weighed to 0.5 g and extracted with 10X volume of extraction buffer (0.1 M NaAscorbate, 0.1 M Bis-Tris, and 5 mM of β-mercaptoethanol) in a 15 mL Falcon tube. The cap of the tube was removed and replaced with a small piece of aluminum foil. The solution was boiled for 15-20 minutes, placed on ice to cool, homogenized, and centrifuged at 5000 rpm at 4 ºC for 20 minutes. The supernatant was collected and stored at -80 ºC. On the day of the folate assay, the liver tissue extract was thawed and mixed with chicken pancreas conjugase and 0.1 M KPO_4 buffer, in a ratio of 4:1:15. The solution was then incubated for 2 hours at 37 ºC, and 125 µL of the liver extract solution was diluted with 900 µL of 0.1 M KPO_4 buffer.

Determination of Plasma and Liver Folate Concentrations:

L. rhamnosus stock (3 µL) was thawed and inoculated in 3 mL of Lactobacillus MRS broth overnight for 16-18 hours at 37 ºC. The following day, 500 µL of the overnight culture was added to 2.5 mL of Lactobacillus MRS broth for 5 hours at 37 ºC. Meanwhile, 0.1 M KPO_4 buffer and folic acid media (9.4 g folic acid media, 100 mL ddH_20, 0.05 g sodium ascorbate, heated to dissolve, filter sterilized) were prepared. A 96-well microtiter plate was obtained and 150 µL of 0.1 M KPO_4 buffer was added to each well. Furthermore, 150 µL of folic acid standard (2 ng/mL) was added to two designated wells serial dilutions were made along the plate in order to create a standard curve. For the plasma folate assay, 5 µL of samples were added, while 10 µL of diluted liver extract samples were used for the liver folate assay. In the wells designated for samples, a sufficient amount of 0.1 M KPO_4 buffer was added in order to bring the total volume to 300 µL in each well (145 µL and 140 µL for the plasma and liver folate assays, respectively). Duplicates of samples were added, and serial dilutions were made along the plate.

When the optical density (OD) reached 1.8, the *L. rhamnosus* inoculum was washed 3 times with folic acid medium. Following this, the *L. rhamnosus* inoculum was diluted 2 times with folic acid medium (24X and then 40X). In each well, 150 µL of the 40X diluted solution was then added. The plate was covered with a mylar sealer, mixed, and incubated at 37 ºC for 16-18 hours. The following day, the plate was read on a spectrophotometer at 650 nm, and the absorption readings in wells were converted to concentrations of folate for each sample, using SoftMax software.

4.2.5 Plasma Homocysteine Concentrations

To determine plasma homocysteine concentrations, the Axis^{TM} Homocysteine EIA kit was used. Before using the kit, all solutions and microtitre strips were equilibrated to room temperature overnight. An hour before commencing the assay, the sample pre-treatment solution was made. The reagents were mixed and the calibrators, samples, and controls were diluted (25 µL calibrator/sample/control + 500 µL of the sample pre-treatment solution). The samples were incubated for 30 minutes at 37 ºC, and were covered with parafilm during incubation. Without allowing the samples to cool, 500 µL of Enzyme Inhibitor (Merthiolate, phosphate buffer) was added to each sample and incubated for 15 minutes at 18-25 ºC. This same amount of Adenosine deaminase (Adenosine deaminase, phosphate buffer, sodium azide, BSA, phenol-red dye) was then added, and incubated for 5 minutes at 18-25 ºC. The diluted calibrator/sample/control (25 µL) was then added to the wells of the SAH-coated microtitre strip-plate, and 200 µL of a-SAH antibody (Monoclonal mouse-anti-S-adenosyl-L-homocysteine antibody, BSA, merthiolate) was added to each well and incubated for 30 minutes at 18-25 ºC. The strip-plate was washed 4 times with 300 µL of diluted wash buffer, and then emptied and dried onto paper towel. In each well, 100 µL of Enzyme conjugate (Rabbit anti-mouse-antibody enzyme conjugate, BSA, horse radish peroxidise, blue dye) was added and was incubated for 20 minutes at 18-25 ºC. The strip-plate was washed again as described above, and 100 µL of Substrate Solution (N-methyl-2-pyrrolidion, propylemeglycol) was then added to each well and incubated for 10 minutes at 18-25 ºC. Lastly, the Stop Solution (0.8 sulphuric acid) was added to each well, the plate was shaken, and the absorbance was read within 15 minutes at 450 nm using a spectrophotometer.

4.2.6 Genomic DNA Isolation and Methylation

DNA Isolation:

Liver stored at -80 ºC was thawed, and 50 mg of liver was homogenized in 700 µL of lysis buffer (100 mM NaCl, 10 mM Tris.Cl pH 8, 25 mM EDTA pH 8, 0.5% SDS). The liver was then digested overnight for 16 hours with 35 µL of proteinase K (20 mg/mL) at 50 ºC with agitation. The following day, 10 µL of RNase A (100 mg/mL) was added and incubated for 1 hour at 50 ºC. Genomic DNA was extracted with phenol, chloroform, and isoamylalcohol, and precipitated with 10% NaOAc and 2 volume of cold 100% ethanol. The sample was gently inverted several times, until visible strands of DNA were observed. The visible, coiled DNA was gently removed, washed with 700 µL of cold 70% ethanol, and centrifuged at 10,000 x g for 1-2 minutes at 18-25 ºC. The 70% ethanol was removed and the purified DNA pellet was air dried for approximately 10 minutes. The purified DNA pellet was then resuspended with 50 µL of TE buffer (10 mM Tris.Cl pH 8, 1 mM EDTA pH). The OD of the samples were measured using a spectrophotometer, and all samples had an absorbance of 260 nm/280 nm ratio between 1.8 and 2.0, indicating high quality DNA that was not contaminated with RNA or protein. The DNA samples were then stored at -4 ºC.

Genomic DNA Methylation:

DNA extracted from liver (0.2 µg/µL) was incubated with 2 µCi of [^{3}H]-methyl-SAM (New England Nuclear, Boston, MA), 3 units of Sss1, and 1X Sss1 methylation buffer (120 mM NaCl, 10 mM Tris.HCl pH 7.9, 10 mM dithiothreitol) in a total volume of 30 µL for 1 hour at 30 ºC. Samples were prepared in duplicate. The mixture was incubated at 65 ºC

for 10 minutes. From each duplicate sample, 12 µL was spotted onto Whatman DE-81 ion exchange filters (Fisher Scientific, Springerfield, NJ) and the filters were dried under incandescent lamps for 12 minutes. Once dry, the filters were washed with fresh sodium phosphate washing buffer (5% Na_2HPO_4) (twice with 20 mL for 20 minutes, followed once 10 mL for 10 minutes). The washed filters were dried under incandescent lamps overnight, and the following day were placed into scintillation vials containing 10 mL of non-aqueous scintillation fluid. Radioactivity was measured using a 1217 RakBeta Scintillation counter (Fisher Scientific, Springerfield, NJ). A control filter (filter with no DNA) was used to assess the background radioactivity and was subtracted from the sample measurements. The spotting and washing was repeated for each sample (2 duplicates); therefore, a total of 4 values were obtained per sample.

4.2.7 Statistical Analysis

The method of Generalized Estimating Equations was used to account for the fact that pups from the same dam are expected to be somewhat more similar to one another than pups from different dams. The maternal diets were measured at the level of the mother, while sex and time of sacrifice were characteristics of the pups that are clustered within their mother. The model included all 2-way interactions between diet, sex, and time.

Due to the fact pups were clustered within each mother, the design effect imposed by the intracluster correlation coefficient (ICC) was taken into account when calculating the sample size. In order to maximize the number of clusters and minimize the cluster size, 4 pups from each mother (2 males and 2 females) were chosen to be randomized. Assuming a small ICC, a total sample size of 160 (10 pups for each of the 16 combinations of diet, sex,

and time) was required to provide 80% power to detect an interaction effect size of 1.4, 1.4, and 0.77 for the 2-way interactions of diet*sex, diet*time, and sex*time, respectively. In the absence of interactions, this also provided 80% power to detect effect sizes of 0.87, 0.50, and 0.50 for the main effects of diet, sex, and time, respectively.

Histograms of each dependent variable were made before statistical testing in order to ensure the data were normally distributed. Data that were not normally distributed were log-transformed in order to obtain normal distribution. All statistical tests were performed using SAS version 9.1 (SAS Institute Inc., Cary, NC) and graphs were prepared in Microsoft Excel. All statistical tests were two-sided at an alpha level of 0.05.

4.3 Results

4.3.1 Actual Number of Mothers and Pup Allocation

Initially, several female mice had difficulty becoming pregnant; therefore, an additional 6 female mice were obtained for breeding. A total of 46 mothers were used to produce pups throughout the duration of the study. The final numbers of mothers assigned to each maternal diet group is shown in **Table 4.4.** From these 46 mothers, a total of 251 pups were randomized. The final allocation of pups is shown in **Tables 4.5-4.6**.

Table 4.4 Final number of mothers used in the experimental study

Maternal Diet (mg/kg diet)	Number of Mothers
2	10
5	10
8	11
25	15
	Total: 46

Table 4.5 Final allocation of pups

	Number of Pups		
Time of Sacrifice	**Male**	**Female**	**Total**
3 Weeks	57	57	114
18 Weeks	68	74	142
Total	125	131	256

Table 4.6 Description of litters

Litter Composition	Number of Mothers (%)
At least 2 female and 2 male pups	83
At least 2 female pups but < 2 male pups	8
< 2 female pups but at least 2 male pups	7
< 2 female pups and < 2 male pups	2

Due to the small size of offspring at 3 weeks of age, and therefore a limited amount of blood and liver tissue obtained at the time of sample collection, not all of the 256 pups were used in each experimental analysis. **Table 4.7** summarizes the total number of pups in each diet/sex/time group (16 groups total) used for plasma folate, liver folate, plasma homocysteine and liver global DNA methylation analysis.

Table 4.7 Total number of pups in each diet/sex/time group used for plasma folate, liver folate, plasma homocysteine (tHcy) and liver global DNA methylation analysis

	Experiment			
Pup Diet/Sex/Time	**Plasma Folate**	**Liver Folate**	**Plasma tHcy**	**Liver Global DNA Methylation**
2 mg M 3 wks	14	9	8	10
2 mg F 3 wks	24	12	7	11
2 mg M 18 wks	14	13	17	14
2 mg F 18 wks	14	12	13	11
5 mg M 3 wks	9	10	6	8
5 mg F 3 wks	11	14	8	8
5 mg M 18 wks	15	13	13	14
5 mg F 18 wks	20	15	20	13
8 mg M 3 wks	14	10	11	8
8 mg F 3 wks	12	8	9	10
8 mg M 18 wks	11	12	12	13
8 mg F 18 wks	15	12	17	14
25 mg M 3 wks	14	14	12	12
25 mg F 3 wks	15	14	10	16
25 mg M 18 wks	19	19	18	12
25 mg F 18 wks	21	19	21	18

*tHcy=homocysteine; M=male; F=female

4.3.2 Litter Size

There was no significant difference in the average litter size between any of the maternal diet groups (p=0.93) **(Figure 4.2)**.

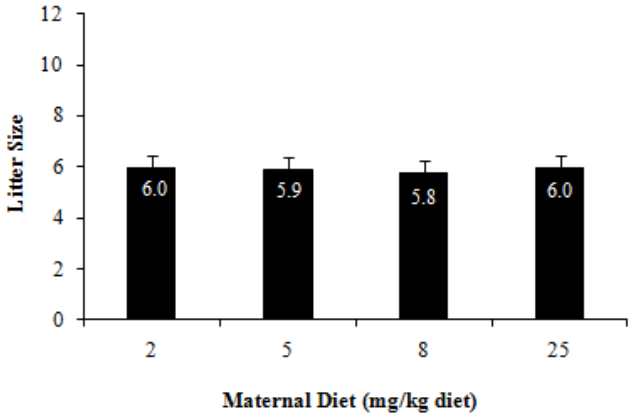

Figure 4.2 Litter size from the 4 maternal diet groups. Values are mean ± SEM**.** The main effect of diet was not significant, p=0.93.

4.3.3 Body Weight at Weaning

The mean body weight of pups at 3 weeks of age did not significantly differ among the 4 maternal diet groups (p=0.16) **(Figure 4.3)**. However, male pups were significantly heavier than female pups, as the main effect of sex was statistically significant (p=0.036). On average, male pups weighed 0.23 g more than female pups at 3 weeks of age. An interaction between maternal diet and pup sex was not statistically significant (p-interaction=0.36).

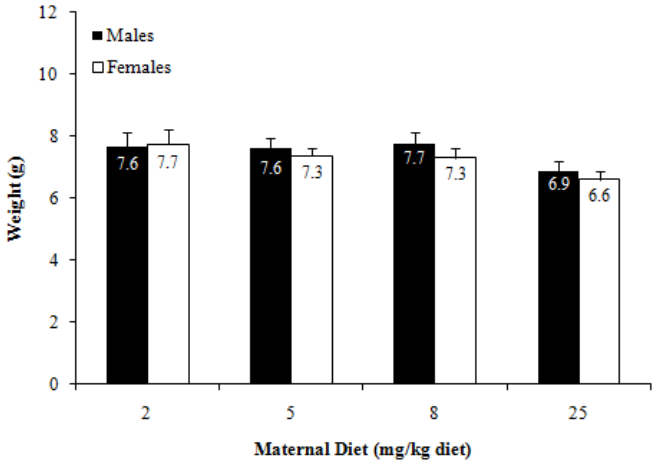

Figure 4.3 Body weights of male and female offspring from the 4 maternal diet groups at 3 weeks of age. Values are mean ± SEM**.** The main effect of diet was not significant, p=0.16. The main effect of sex was significant, p=0.036.

4.3.4 Body Weight Over Time

Maternal diet did not significantly affect the rate of weight gain from 3 weeks to 18 weeks of age (p=0.87) **(Figure 4.4)**. However, male pups increased their weight at a significantly greater rate than female pups, as the main effect of sex was statistically significant (p<0.0001). On average, male pups gained 6.6% each week, while female pups gained only 4.8% each week. An interaction between maternal diet and pup sex was not statistically significant (p-interaction=0.87).

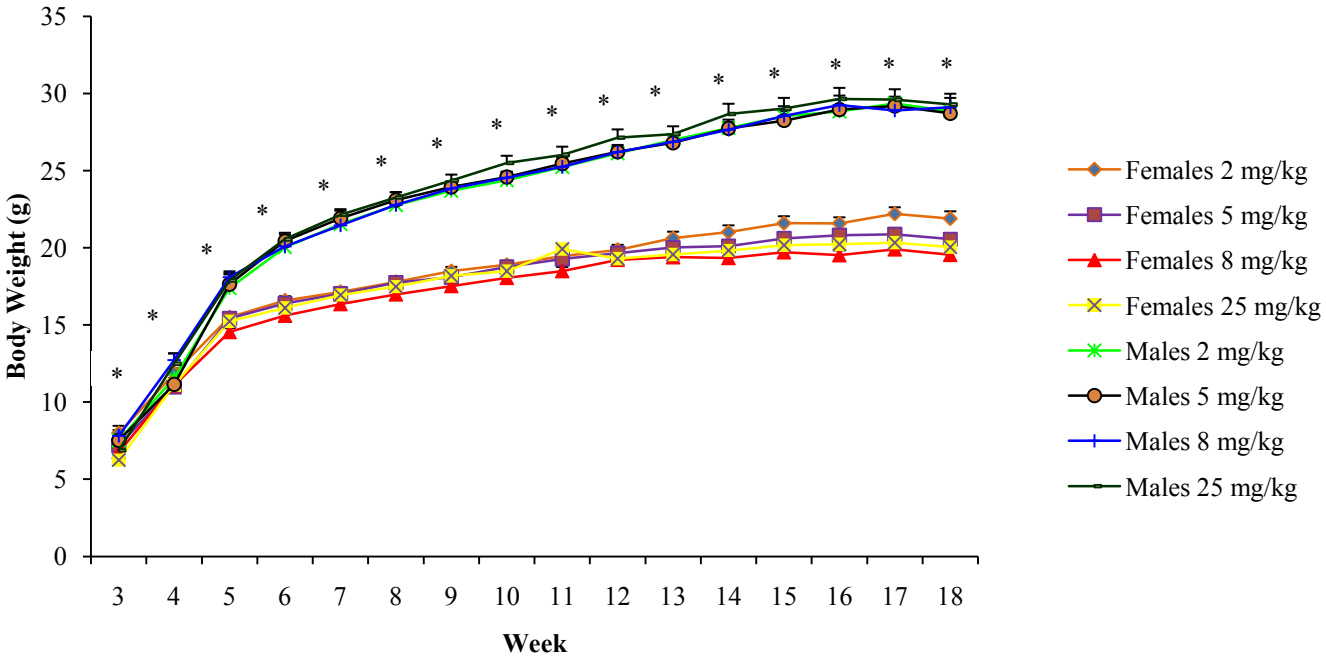

Figure 4.4 Weekly body weights (from 3 weeks until 18 weeks of age) of male and female offspring from the 4 maternal diet groups. Values are mean ± SEM. The main effect of diet was not significant, p=0.87. Asterisks indicate means between males and females at each time point significantly differ, p<0.0001.

4.3.5 Plasma Folate Concentrations

In mice 3 weeks of age, mean plasma folate concentrations were significantly higher in pups from the 5, 8, and 25 mg folic acid/kg diet maternal diet groups compared with that of pups from the maternal control diet ($p<0.05$) **(Figures 4.5-4.6)**. In mice 18 weeks of age, mean plasma folate concentrations were significantly higher in pups from the 8 and 25 mg folic acid/kg diet maternal diet groups compared with that of pups from the maternal control diet ($p<0.05$), while no significant difference was observed between the control and 5 mg folic acid/kg diet maternal diet groups.

Male pups had significantly higher plasma folate concentrations than female pups, as the main effect of sex was statistically significant (p=0.0033). On average, plasma folate concentrations were 4.67 ng/mL higher in males than females.

A significant interaction was detected between maternal diet and time (p-interaction=0.0015), indicating that the main effect of maternal diet on plasma folate concentrations in the offspring depended on time, and similarly, the main effect of time on plasma folate concentrations in the offspring depended on which diet the mother was on.

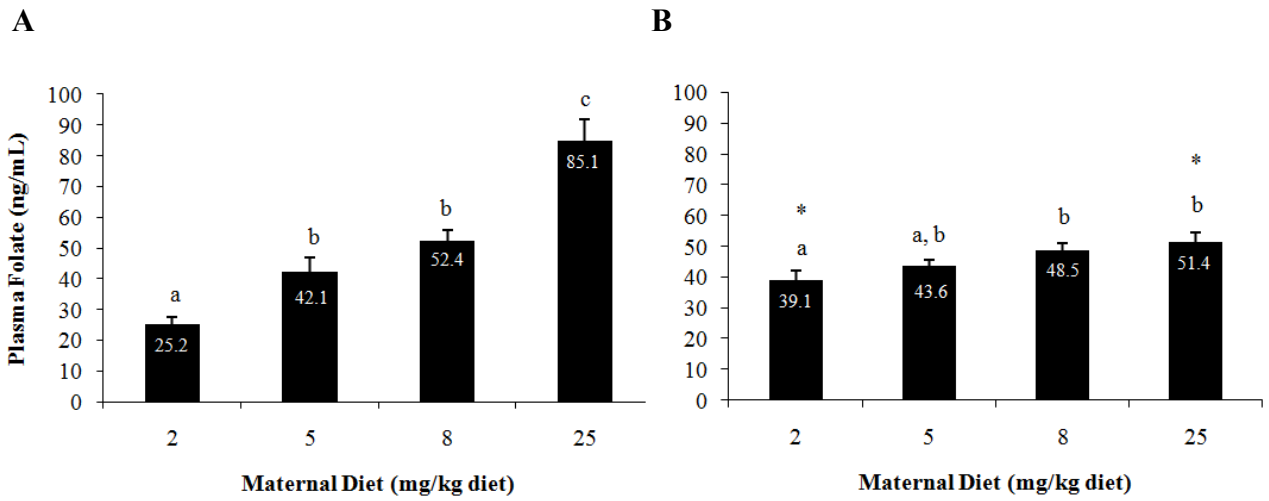

Figure 4.5 Plasma folate concentrations in male offspring from the 4 maternal diet groups at (**A**) 3 weeks of age and (**B**) 18 weeks of age. Values are mean ± SEM**.** Means without a common letter within each time point significantly differ, p<0.05. Asterisk indicates mean is significantly different from that at 3 weeks of age for each maternal diet group, p<0.05.

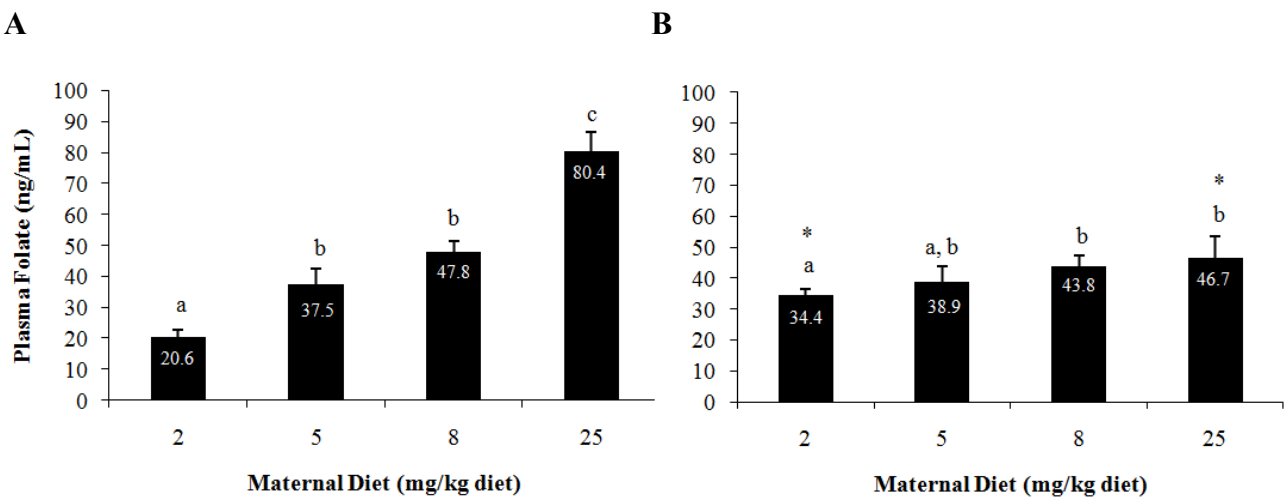

Figure 4.6 Plasma folate concentrations in female offspring from the 4 maternal diet groups at (**A**) 3 weeks of age and (**B**) 18 weeks of age. Values are mean ± SEM**.** Means without a common letter within each time point significantly differ, $p<0.05$. Asterisk indicates mean is significantly different from that at 3 weeks of age for each maternal diet group, $p<0.05$.

4.3.6 Liver Folate Concentrations

At 3 weeks of age, mean liver folate concentrations were significantly higher in pups from the 5, 8, and 25 mg folic acid/kg diet maternal diet groups compared with that of pups from the maternal control diet ($p<0.05$) (**Figures 4.7-4.8**). However, by 18 weeks of age, mean liver folate concentrations did not differ significantly among offspring from any of the maternal diet groups ($p>0.05$). In addition, a significant increase in mean liver folate concentrations was observed from 3 weeks to 18 weeks in offspring from each of the 4 maternal diet groups ($p<0.0001$).

The main effect of sex was not statistically significant ($p=0.79$), indicating that liver folate concentrations did not significantly differ between male and female offspring.

A significant interaction was detected between maternal diet and time (p-interaction<0.0001), indicating that the main effect of maternal diet on liver folate

concentrations in the offspring depended on time, and similarly, the main effect of time on liver folate concentrations in the offspring depended on which diet the mother was on.

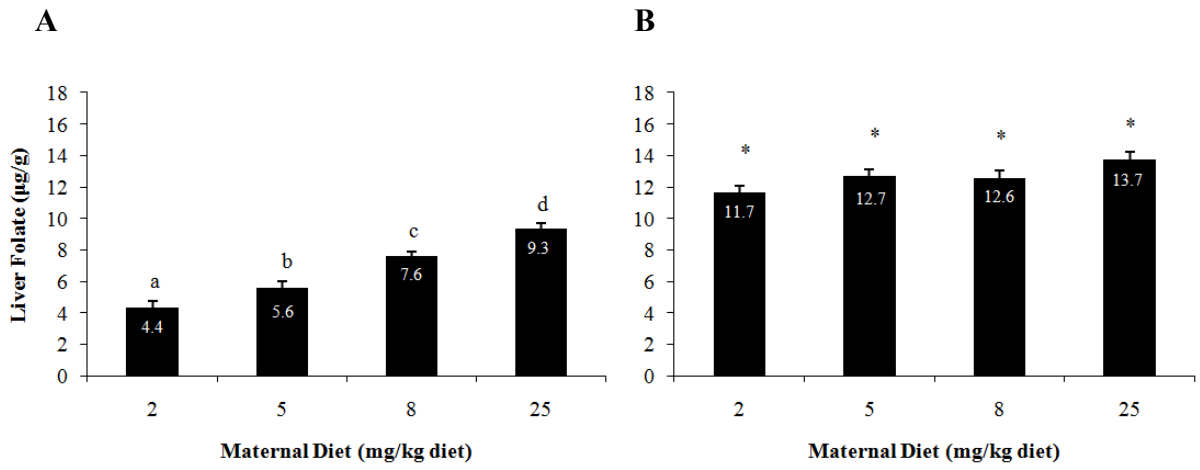

Figure 4.7 Liver folate concentrations in male offspring from the 4 maternal diet groups at (**A**) 3 weeks of age and (**B**) 18 weeks of age. Values are mean ± SEM**.** Means without a common letter within each time point significantly differ, $p<0.05$. Asterisk indicates mean is significantly different from that at 3 weeks of age for each maternal diet group, $p<0.05$.

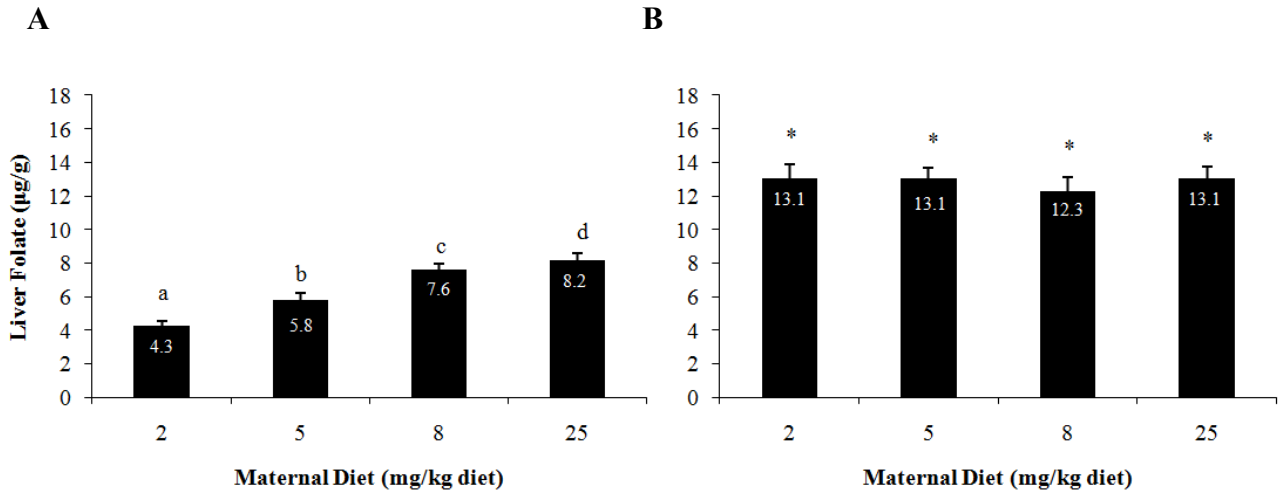

Figure 4.8 Liver folate concentrations in female offspring from the 4 maternal diet groups at (**A**) 3 weeks of age and (**B**) 18 weeks of age. Values are mean ± SEM**.** Means without a common letter within each time point significantly differ, $p<0.05$. Asterisk indicates mean is significantly different from that at 3 weeks of age for each maternal diet group, $p<0.05$.

4.3.7 Plasma Homocysteine Concentrations

At 3 and 18 weeks of age, mean plasma homocysteine concentrations of the offspring did not significantly differ among the 4 maternal diet groups ($p>0.05$) (**Figures 4.9-4.10**).

The main effect of time was statistically significant, indicating that plasma homocysteine concentrations in the offspring significantly decreased from 3 to 18 weeks of age ($p=0.0012$). On average, mean homocysteine concentrations were 3.21 μmol/L higher in offspring at 3 weeks of age compared to offspring at 18 weeks age.

Although not statistically significant, there was a trend for an interaction between maternal diet and time was observed (p-interaction=0.083). Thus, the main effect of maternal diet on plasma homocysteine concentrations in the offspring may depend on time, and similarly, the main effect of time on plasma homocysteine concentrations in the offspring may depend on which diet the mother was on. In addition, there was a nonsignificant trend for an interaction between pup sex and time was observed (p-interaction=0.067). Therefore, the main effect of pup sex on plasma homocysteine concentrations in the offspring may depend on time, and similarly, the main effect of time on plasma homocysteine concentrations in the offspring may depend on pup sex.

In addition, plasma homocysteine concentrations were significantly negatively correlated with liver folate concentrations ($r=-0.20$, $p=0.0084$).

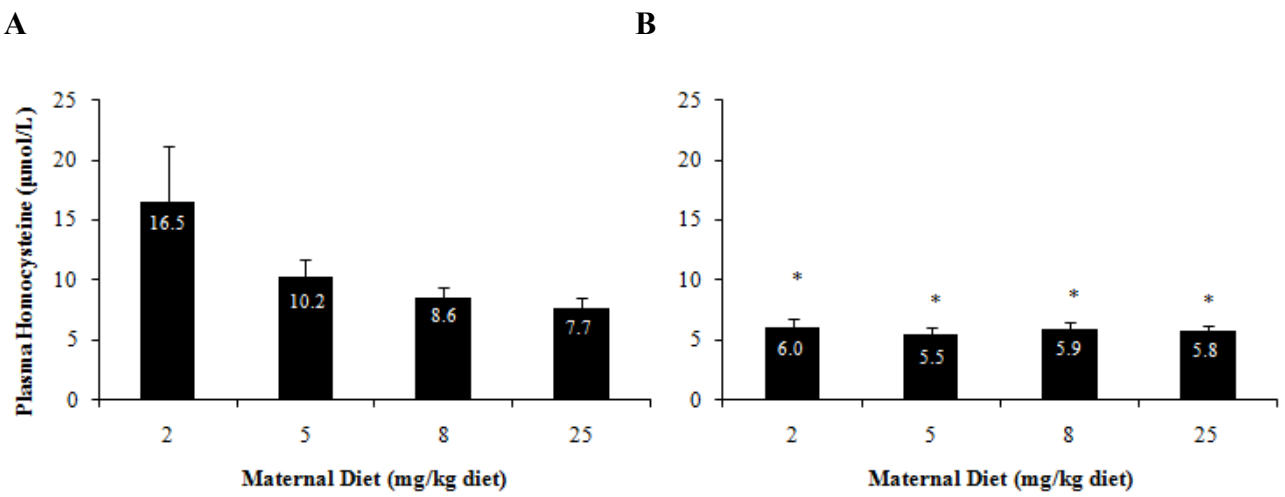

Figure 4.9 Plasma homocysteine concentrations in male offspring from the 4 maternal diet groups at (**A**) 3 weeks of age and (**B**) 18 weeks of age. Values are mean ± SEM**.** Asterisk indicates mean is significantly different from that at 3 weeks of age for each maternal diet group, $p<0.05$.

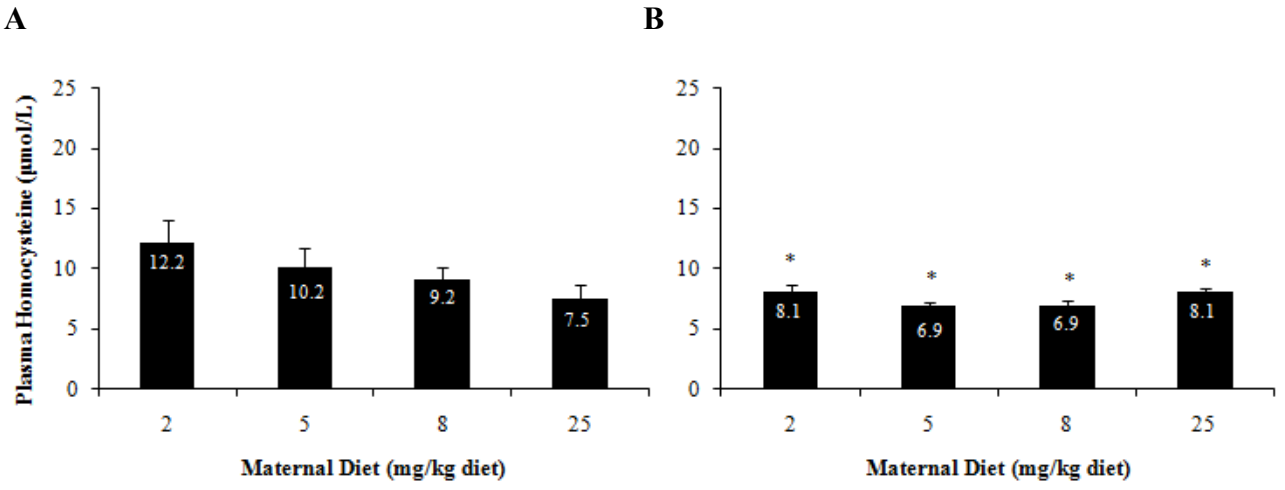

Figure 4.10 Plasma homocysteine concentrations in female offspring from the 4 maternal diet groups at (**A**) 3 weeks of age and (**B**) 18 weeks of age. Values are mean ± SEM**.** Asterisk indicates mean is significantly different from that at 3 weeks of age for each maternal diet group, $p<0.05$.

4.3.8 Genomic DNA Methylation

Global DNA methylation in the liver was measured using the *in vitro* methyl acceptance capacity assay that incorporates radio-labelled ^{3}H-methyl groups into genomic DNA; therefore, a reciprocal relationship exists between the endogenous DNA methylation status and exogenous ^{3}H-methyl incorporation into DNA.

At 3 weeks of age, liver global DNA methylation was significantly lower in offspring from the 5, 8, and 25 mg folic acid/kg diet maternal diet groups compared with offspring from the maternal control group ($p<0.05$) (**Figures 4.11-4.12**). At 18 weeks of age, liver global DNA methylation was significantly lower in offspring from the 5, 8, and 25 mg folic acid/kg diet maternal diet groups compared with offspring from the maternal control group ($p<0.05$).

The main effect of time was not statistically significant, indicating that ^{3}H-methyl incorporation into hepatic DNA did not differ between 3 and 18 weeks of age ($p=0.56$). Therefore, the effects of maternal folic acid supplementation on global DNA methylation persisted into adulthood.

In addition, ^{3}H-methyl incorporation into hepatic DNA was positively correlated with plasma folate concentrations and liver folate concentrations ($r=0.18$, $p=0.023$ and $r=0.16$, $p=0.04$, respectively), while ^{3}H-methyl incorporation into hepatic DNA was not correlated with plasma homocysteine concentrations ($r=-0.03$, $p=0.67$).

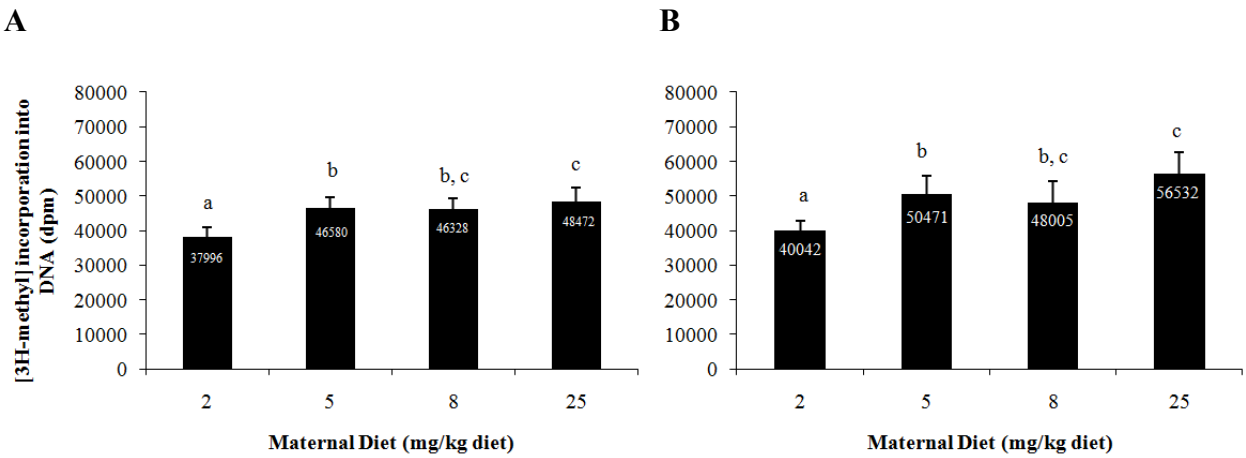

Figure 4.11 Global DNA methylation in the liver of male offspring from the 4 maternal diet groups at (**A**) 3 weeks of age and (**B**) 18 weeks of age. Values were determined by the *in vitro* methyl acceptance assay. A reciprocal relationship exists between the endogenous DNA methylation status and the exogenous ^{3}H-methyl incorporation into DNA. Values are mean ± SEM**.** The exogenous ^{3}H-methyl incorporation into DNA was measured on the log scale, while the above figure presents the results on the original scale. Means without a common letter within each time point significantly differ, $p<0.05$.

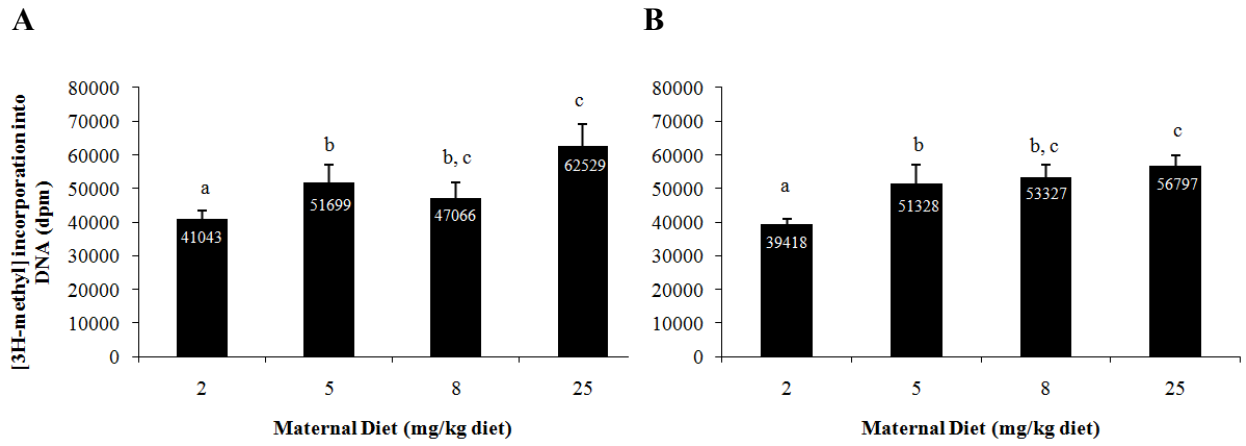

Figure 4.12 Global DNA methylation in the liver of female offspring from the 4 maternal diet groups at (**A**) 3 weeks of age and (**B**) 18 weeks of age. Values were determined by the *in vitro* methyl acceptance assay. A reciprocal relationship exists between the endogenous DNA methylation status and the exogenous ^{3}H-methyl incorporation into DNA. Values are mean ± SEM**.** The exogenous ^{3}H-methyl incorporation into DNA was measured on the log scale, while the above figure presents the results on the original scale. Means without a common letter within each time point significantly differ, $p<0.05$.

4.4 Discussion

Emerging evidence suggests that nutritional alterations during the earliest stages of life, such as *in utero* and/or the early postnatal period, may alter DNA methylation patterns in the offspring [21, 24, 31]. For example, an epigenetic effect of maternal diets supplemented with methyl donors and their cofactors (methionine, choline, folic acid, and vitamin B_{12}) has been shown in the *agouti* mouse [21, 226]. However, these studies, in addition to numerous other studies, do not address the consequences of maternal folic acid supplementation alone. Thus, the primary aim of the present study was to investigate the effect of maternal folic acid supplementation *in utero* and during lactation on global DNA methylation in the liver of both juvenile and adult offspring, using supplemental levels of folic acid equivalent to 2X, 4.5X, and 12.5X the RDA in humans – levels which reflect the amount of folic acid that many women of childbearing age are consuming in the post-fortification era. Due to the essential role of folate in DNA methylation, we hypothesized that intrauterine and early postnatal exposure to supplemental levels of folic acid would increase global DNA methylation in the liver of juvenile offspring. Furthermore, we hypothesized that this hypermethylation would persist into adulthood, after new DNA methylation patterns were established *in utero* in response to supplemental levels of folate. We observed that maternal folic acid supplementation of 5, 8, and 25 mg folic acid/kg diet induced significant global DNA hypomethylation in the liver of offspring at 3 weeks of age, and that this effect persisted into adulthood.

The results from the present study demonstrate that maternal folic acid supplementation of varying doses had no effect on litter size or body weight of the offspring at 3 weeks of age. Due to the fact weight at birth was not measured in the present study, it is

possible that body weight at 3 weeks of age may not accurately reflect body weight at birth. However, similar to the present study, Engeham *et al*. have recently reported that maternal folic acid supplementation (5 mg folic acid/kg diet) throughout pregnancy was shown to have no effect on litter size or fetal and young offspring body weight in rats [32]. In that study as well as the present study, it is possible that there were no differences observed in offspring body weight due to the fact the size of the litters did not vary between any of the maternal diet groups.

However, several other recent animal studies, including two studies from our laboratory, found significant differences in offspring body weight at 3 weeks of age following maternal folic acid supplementation of a moderate degree, despite no significant differences in litter size among the maternal diet groups. In one study, maternal folic acid supplementation (5 mg folic acid/kg diet) throughout pregnancy and lactation was shown to increase the body weight of offspring at 3 weeks of age [138]. In contrast, Sie *et al*. found that this same degree of maternal folic acid supplementation in rats decreased the body weight of female offspring at 3 weeks of age [131]. In another study, maternal folic acid supplementation (40 mg folic acid/kg diet) for 3 weeks in pregnant rats was shown to decrease body weight in fetuses compared to those from the control maternal diet group [258]. Similarly, Szeto *et al*. have recently shown that high multivitamin intake (10X the amount of multivitamin as the regular diet AIN-93G) throughout pregnancy had no effect on litter size, while at weaning, body weights of offspring from the maternal high multivitamin group were significantly lower than offspring from the maternal control group [259].

In humans, a positive association between increased maternal folic acid intake, higher birth weight, and a reduced risk of low birth weight has been suggested [260-262]. In a more

recent prospective-population based cohort study, low-dose periconceptional folic acid supplementation was associated with an increase in fetal growth resulting in higher placental and birth weight, and a decreased risk of having a child with low birth weight compared to non-supplemental users [263]. Furthermore, in the Pune Maternal Nutrition Study, maternal RBC folate concentrations at 28 weeks gestation were positively associated with birth weight, birth length, head circumference, mid-upper-arm circumference, abdominal circumference, and placental weight [264].

In the present study, we also found that maternal diet had no effect on the rate of weight gain in the offspring from 3 to 18 weeks of age. In rats, Ly *et al*. observed that offspring from mothers supplemented with 5 mg folic acid/kg diet throughout pregnancy and lactation weighed significantly more than those from the maternal control diet group; however, this difference decreased over time and disappeared by week 10 [138].

The inconsistent results from studies examining the effect of maternal folic acid supplementation on body weight in the offspring may be due differences among species. For example, in the above mentioned animal studies, rats were utilized; therefore, the inconsistent results between the present study and these animal studies may be due to differences between mice and rats. In mice, it has previously been shown that maternal dietary supplementation with very high levels of methyl donors (including folic acid) had no effect on litter size or offspring body weight at postnatal day 21 in the A^{vy} mouse model [21]. In addition, food intake was not measured in some of these animal studies and therefore may have been different among the diet groups.

In the present study, plasma folate concentrations in the offspring from mothers supplemented with 5 mg folic acid/kg diet appeared to be slightly lower than values obtained

previous studies from our laboratory. Due to the fact the timing and duration of the maternal diets in these studies and the present study were similar, it is likely that the differences observed in plasma folate concentrations are due to differences between species. Plasma folate levels in offspring from the maternal 8 mg folic acid/kg diet group in the present study are comparable to values from several other studies using this level of folic acid supplementation; however, in those studies, folic acid supplementation was initiated at weaning or later on in life [25, 169]. To our knowledge, no studies at present have measured plasma folate levels in rodents following a 25 mg folic acid/kg diet. However, Kim *et al*. demonstrated that in rats fed a diet containing 0, 2, 8, or 40 mg folic acid/kg diet for 20 weeks, plasma folate concentrations differed among the diet groups in a dose-dependent manner, and plasma folate concentrations reported in that study were comparable to values in the present study [173]. In addition, two recent studies have reported extremely high levels of plasma folate (>200 ng/mL) following a folic acid supplemented diet containing 40 mg/kg folic acid initiated at weaning and at 5 weeks of age [169, 180].

Plasma folate concentrations are an accurate indicator of systemic folate status, and are affected by recent dietary intake [49]. In the present study, plasma folate concentrations in offspring from mothers supplemented with folic acid were significantly higher at 3 weeks of age compared to offspring from the maternal control group, and this effect appeared to be dose-dependent. Due to the fact the offspring consumed the control diet from 3 weeks of age and onwards, plasma folate concentrations in offspring from mothers consuming the folic acid supplemented diets were expected to be similar to those values of offspring from the maternal control group at 18 weeks of age. Interestingly, adult offspring from the maternal 8 and 25 mg folic acid/kg diet groups had significantly higher levels of plasma folate compared

to adult offspring from the maternal control group. It is unknown why plasma folate concentrations in these groups remained significantly higher than the control; however, this was also recently demonstrated in a study from our laboratory in offspring from mothers supplemented with 5 mg folic acid/kg diet throughout pregnancy and lactation [131].

Liver tissue folate concentrations were also measured in the present study, and the values obtained were comparable to those reported in previous rodent studies from our laboratory [131, 138]. Similar to plasma folate concentrations, liver folate concentrations appeared to increase with maternal folic acid supplementation in a dose-dependent manner in offspring at 3 weeks of age. In addition, liver folate concentrations significantly increased from 3 to 18 weeks of age. In humans, total fetal folate stores appear to be substantially lower than hepatic folate concentrations in adults [63]. Fetal hepatic folate concentrations have been shown to range from 1.5 to 4.0 μg/g [265, 266], while adult hepatic folate concentrations have been shown to reach concentrations greater than 5.0 μg/g [267, 268]. Therefore, it is possible that fetal and early postnatal folate acquisition and utilization differs from those of adults. At 18 weeks of age, liver folate concentrations in the present study did not significantly differ among offspring from the 4 maternal diet groups. These observations were expected, as the control diet was initiated at the time of weaning, and therefore, offspring were not exposed to supplemental levels of folic acid beyond this time point.

In adults, dietary folate intake has been shown to be one of the major determinants of blood homocysteine concentrations, and supplementation with folic acid has been shown to effectively reduce homocysteine concentrations [269-271]. In a recent study in humans, the effectiveness of 0.2 mg folic acid/day with that of 0.4 and 0.8 mg folic acid/day at lowering homocysteine concentrations was examined over a 6 month period. Interestingly, a dose of

0.2 mg folic acid/day administered for 6 months was shown to effectively lower homocysteine concentrations, while higher doses did not further lower homocysteine levels [272].

Unexpectedly, maternal folic acid supplementation of increasing doses failed to significantly modulate plasma homocysteine levels in offspring at 3 and 18 weeks of age in the present study. In contrast, a significant decrease in plasma homocysteine concentrations in the offspring following maternal folic acid supplementation *in utero* and during lactation have been observed in previous studies from our laboratory [131, 138]. However, the results from the present study do suggest a nonsignificant, slight decrease in plasma homocysteine in offspring at 3 weeks of age. It is important to note that due to the small body size and therefore limited amounts of blood obtained at the time of sacrifice from mice at 3 weeks of age, a relatively small number of samples were analyzed for plasma homocysteine. Therefore, this would have contributed to loss of power to detect an effect. It is possible that a larger sample size would have shown a significant effect of maternal folic acid supplementation on plasma homocysteine.

However, a recent study in rats found that maternal folic acid supplementation (5 mg folic acid/kg diet) throughout pregnancy did not alter fetal homocysteine levels at gestational day 20 [32]. It is possible that homocysteine metabolism in the newborn may differ from that later in life. This is supported in humans by a recent prospective randomized intervention study in The Netherlands, which demonstrated that daily administration of 70 μg/kg of 5-formylTHF (folinic acid), an active form of folate, either intravenously or orally for 2 weeks in newborns had no effect on plasma homocysteine concentrations [273]. Furthermore, in 173 healthy newborns at 4 days of age, no significant association between

serum or whole blood folate and homocysteine was observed, while vitamin B_{12} status appeared to be the main determinant of plasma homocysteine [274].

Global DNA methylation in the present study was measured in the liver of offspring at 3 and 18 weeks of age, and was determined by the *in vitro* methyl acceptance assay. Due to the role of folate in DNA methylation, we expected to observe an increase in global DNA methylation in offspring liver at 3 weeks of age. Unexpectedly, however, we observed a significant degree of global DNA hypomethylation in offspring from mothers that consumed the folic acid supplemented diets compared to offspring from the maternal control group at 3 weeks of age. However, we did expect the DNA methylation patterns established during the earliest stages of life to persist into adulthood. Indeed, we observed a significant degree of global DNA hypomethylation in offspring at 18 weeks of age from mothers that consumed the folic acid supplemented diets compared to offspring from the maternal control group. Furthermore, the effect of maternal folic acid supplementation on global DNA methylation at both time points in appeared to be dose-dependent.

The results from this study provide additional evidence to the growing body of literature that suggests the epigenome during the earliest stages of life is highly sensitive to environmental insults, including dietary factors. Supplementing the maternal diet with methyl donors, including folic acid, has been shown to increase DNA methylation of CpG sites at the promoter region of the *agouti* gene, and numerous other animal studies using a variety of maternal diets have also reported alterations in DNA methylation in the offspring [21-24, 31].

Although many studies have examined the effect of folate deficiency on DNA methylation, few studies have examined the effect of isolated folic acid supplementation on

DNA methylation, and the majority of these have investigated the effect of folic acid supplementation initiated after weaning and continued through adulthood. Most of these studies have found that dietary folate supplementation at both moderate and supranormal levels initiated at weaning or later in life did not alter global DNA methylation in rat liver and colon [19, 25, 169, 180]. It is possible that folic acid supplementation had no effect on global DNA methylation in these studies due to the timing of the dietary intervention. The postweaning period may be beyond the window of opportunity in which dietary folate can modulate DNA methylation patterns. Interesting, global DNA methylation in the liver and colon of the elderly has been shown to increase following folic acid supplementation [165, 182]. Therefore, the elderly may be an age group that is particularly susceptible to the modulatory effect of folate on DNA methylation. These studies and our present data collectively suggest that the intrauterine and early postnatal stages and aging might be highly epigenetically susceptible periods.

In contrast to our findings in the present study, Engeham *et al*. recently found that global DNA methylation was not altered in the rat liver of fetusus (gestational day 20) following maternal folic acid (5 mg folic acid/kg diet) supplementation during pregnancy [32]. In recent study from our laboratory, maternal folic acid supplementation during pregnancy and lactation (5 mg folic acid/kg diet) was shown to increase global DNA methylation in the colonic mucosa in offspring at 3 weeks of age, but had no effect on colonic global DNA methylation in offspring at 14 weeks of age [240]. However, the postweaning diet of the offspring did alter global DNA methylation, as pups consuming a diet supplemented with folic acid (5 mg folic acid/kg diet) from weaning until 14 weeks of age demonstrated a significant 3-7% decrease in colonic global DNA methylation compared

to those pups that consumed the control diet. The inconsistent findings between these studies and the present study may be due to the different duration of diets as well as the different tissues examined.

Several recent studies have produced similar findings to those from the present study. Ly *et al*. found that maternal folic acid supplementation (5 mg folic acid/kg diet) throughout pregnancy and lactation in rats induced a significant degree of global DNA hypomethylation in the non-neoplastic mammary gland of offspring at 7 weeks of age [138]. In that study, offspring were placed on either the control or folic acid-supplemented diet at weaning; however, postweaning folic acid supplementation had no effect on global DNA methylation in non-neoplastic mammary glands. Furthermore, maternal folic acid supplementation (5 mg folic acid/kg diet) throughout pregnancy and lactation was shown to induce a significant degree of global DNA hypomethylation in the non-neoplastic mammary gland of offspring at 21 weeks of age who consumed the control diet or a folic acid supplemented diet (5 mg folic acid/kg diet) beginning at weaning. In contrast, maternal folic acid supplementation had no effect in offspring bearing at least one mammary adenocarcinoma.

In humans, Quinlivan *et al*. recently found a modest reduction in genomic DNA methylation following folic acid supplementation [203]. More specifically, in women of reproductive age, genomic DNA methylation decreased by 13% following 1 month of folic acid supplementation (100, 400, 4000 μg/day); however, genomic DNA methylation did not change further during the remainder of the intervention (at 3 and 6 months).

One possibility for the observed global DNA hypomethylation in these studies as well as the present study may be due to upregulation of DHFR. While folic acid may inhibit DHFR [275], it has also been shown to upregulate DHFR [276]. Since transcription of DHFR

and TS is co-regulated by several transcriptional factors [277], an upregulation of DHFR would also increase TS activity and therefore thymidylate production, increasing cellular proliferation at the expense of methylation reactions [278]. Thus, the flux of one-carbon units may be shuttled toward the nucleotide synthesis pathway over the methylation pathway in response to folic acid supplementation.

In the present study, we demonstrated that the effects of maternal folic acid supplementation on DNA methylation were not only observed at 3 weeks of age, but persisted into adulthood. Similarly, in humans, Heijmans *et al*. recently found that individuals who were prenatally exposed to famine *in utero* had, decades later, alterations in DNA methylation [231].

Although we did not investigate the functional ramifications of the observed global DNA hypomethylation in the offspring associated with maternal folic acid supplementation in the present study, the long-term consequences of global DNA hypomethylation could be potentially detrimental. In normal cells, the bulk of the genome is typically heavily methylated, preventing the transcription of large parts of the genome that consist of repeat DNA elements, inserted viral sequences, and transposons [147]. Transposons are sequences of DNA that are capable of moving from their usual location into a new region of the genome [279]. Transposons and endogenous retroviruses are littered throughout the genome and account for more than 35% of the human genome. Since parasitic DNA elements are capable of mediating recombination between non-allelic repeats, causing chromosome rearrangements or translocations or directly disrupting genes, they may be a significant threat to the structural integrity of the genome [280]. These parasitic sequences have strong promoters, which could result in internal initiation if they are integrated within a

transcriptional unit. Furthermore, if integrated in the 'antisense' orientation relative to the normal direction of transcription of the targeted gene, this could inhibit the expression of the gene through transcriptional interference or an antisense mechanism.

Given the observed global DNA hypomethylation in the offspring associated with maternal folic acid supplementation, there remains the possibility that maternal folic acid supplementation may also affect DNA methylation in the promoter CpG islands or CpG sites of many genes in the offspring. Previous animal studies suggest that the direction of changes in global and gene-specific DNA methylation as a result of alterations in dietary folate may not always be in the same direction and the effect of folate deficiency on DNA methylation has been shown to be gene- and site-specific [18, 25, 163]. If maternal folic acid supplementation methylates the CpG promoter islands of tumor suppressor genes and other critical cancer-related genes that are involved in cell cycle control, repair of DNA damage, apoptosis, and differentiation, this would inactivate these genes, thereby contributing to tumor development.

Maternal folic acid supplementation could also affect additional epigenetic modifications in the offspring which have the ability to alter transcriptional activity, including histone modifications and miRNAs. Furthermore, since folate plays an essential role in the nucleotide synthesis pathway, there remains the possibility that maternal folic acid supplementation could affect DNA synthesis, replication, and repair in the offspring.

4.5 Conclusion

Notwithstanding the limitations of the present study, our data suggests that *in utero* and early life exposure to supplemental levels of folic acid, which reflect the amount of folic

acid that many women of childbearing age are consuming in the post-fortification era, is capable of decreasing global DNA methylation in the liver of juvenile offspring, and that this global DNA hypomethylation persists into adulthood. Future studies are required in order to determine whether these effects are tissue-, gene-, and site-specific, whether alterations in DNA methylation vary according to the timing and duration of folic acid supplementation during pregnancy and lactation, and whether these effects may be associated with detrimental functional consequences in the offspring.

Chapter 5: THE EFFECTS OF MATERNAL FOLIC ACID SUPPLEMENTATION PROVIDED IN UTERO AND DURING LACTATION ON GLOBAL GENE EXPRESSION IN THE OFFSPRING

5.1 Introduction

A growing body of evidence suggests that alterations in the maternal diet can induce permanent changes in the phenotype of the offspring, which may affect the risk of developing disease later in life. Despite the beneficial effects of periconceptional folic acid supplementation on NTDs in the offspring, it has recently been suggested that high maternal folate status may have potential adverse effects on the health of the offspring. For example, high maternal folate status may increase the risk of cancer, obesity, insulin resistance, and asthma in the offspring [15, 16, 139]. The biological mechanisms by which cues about nutrient availability at the earliest stages of life are transmitted to the developing organism and the manner in which different phenotypes are induced are largely unknown at present. However, persistent changes in the phenotype must ultimately imply stable changes in gene transcription.

Alterations in folate status have been shown to cause various genetic and epigenetic aberrations, including uracil misincorporation, genomic and gene-specific DNA breaks, and changes in DNA methylation [281-283]. These deleterious genetic and epigenetic changes, as well as others, as a result of modulations in dietary folate, could alter the expression of certain genes. To date, numerous *in vitro*, animal, and human studies have demonstrated that folate deficiency and supplementation are capable of modulating gene expression patterns [25-29]. Less certain, however, is whether intrauterine and early postnatal exposure to high levels of folate can modulate gene expression patterns in the offspring.

Several recent studies have examined the effect of maternal folate supplementation on gene expression in the offspring, although most of these studies have included other dietary components in combination with supplemental levels of folic acid, such as dietary protein restriction [14, 31]. Furthermore, most studies to date have taken a candidate gene approach, while few have examined the effect of maternal folic acid supplementation on global gene expression patterns in the offspring.

To our knowledge, no studies at present have examined the effect of isolated maternal folic acid supplementation of varying doses, including very high levels reflecting the recent SOGC and Motherisk recommendation in humans for women of childbearing age, on global gene expression in the offspring of a mouse model. Thus, the objective of this study is to investigate the effect of maternal folic acid supplementation (5, 8 and 25 mg folic acid/kg diet) provided *in utero* and during lactation on hepatic global gene expression in juvenile offspring. Based on previous studies that have demonstrated that a moderate degree of maternal folic acid supplementation can induce changes in the expression of a variety of genes in the offspring [30, 254], we hypothesize that maternal folic acid supplementation of increasing doses provided during pregnancy and lactation would alter the expression of numerous genes in the offspring.

5.2 Materials and Methods

5.2.1 Experimental Design

The experimental design can be found in ***Chapter 4.2.1***. Due to cost, liver global gene expression was measured only in male offspring at 3 weeks of age. In order to select samples to be used for the microarray experiment, the liver folate concentration of each male pup was compared to the mean value of liver folate concentrations for their respective diet group. Male pups with similar liver folate values to the mean value for each diet group were chosen for the microarray experiment. For each of the 4 maternal diet groups, 2 male pups were chosen for the analysis (total sample size=8).

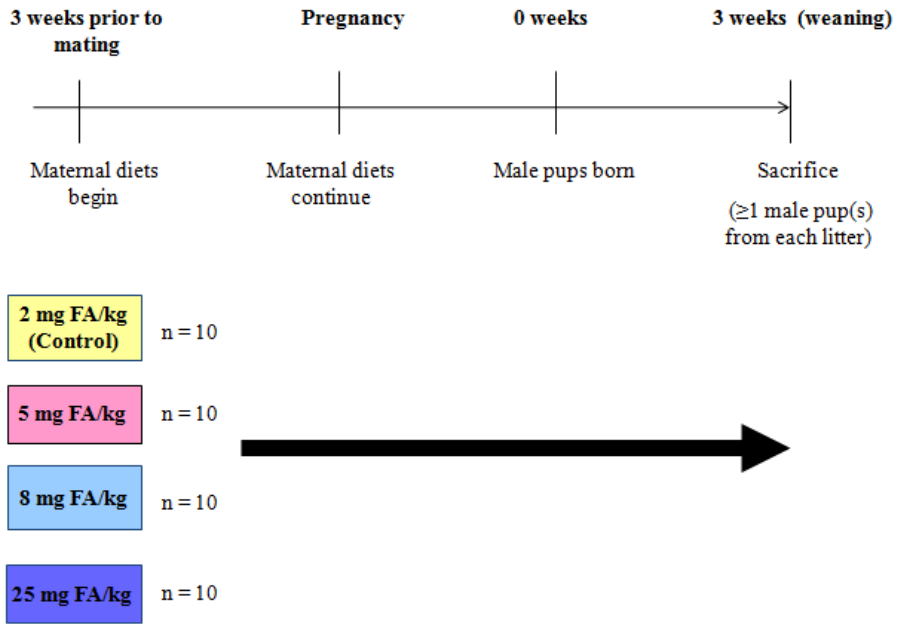

Figure 5.1 Experimental study design. FA=folic acid.

5.2.2 Experimental Diets

Mice were fed L-amino-acid defined diets in pellet form (Dyets, Bethlehem, PA) as previously described in ***Chapter 4.2.2***.

5.2.3 Sample Collection

The sample collection was performed as outlined in ***Chapter 4.2.3***.

5.2.4 Liver Folate Concentrations

Liver folate concentrations were determined using a standard microbiological *Lactobacillus rhamnosus* microtiter plate technique as previously described in ***Chapter 4.2.4***.

5.2.5 Microarray Analysis

RNA Extraction:

RNA was isolated using the Qiagen RNeasy Microarray Tissue Mini Kit, according to the manufacturer's protocol. Liver tissue (10 mg) was homogenized in 1 mL QIAzol lysis reagent (a monophasic solution of phenol and guanidine thiocyanate), and the homogenate was then incubated at 15-25 ºC for 5 minutes. Following the incubation, 200 µL of chloroform was added, the homogenate was shaken vigorously for 15 seconds, and incubated again at 15-25 ºC for 2-3 minutes. The sample was then centrifuged at 12,000 x g for 15 minutes at 4 ºC, the upper aqueous phase was collected, and 1 volume of 70% ethanol was

added to provide appropriate binding conditions. The sample (up to 700 μL) was then transferred to an RNeasy spin column, where total RNA was able to bind to the membrane, and phenol and other contaminants were washed away. RNA was then eluted in 50 μL RNase-free water. The concentration was then measured using a spectrophotometer. All samples had a 260 nm/280 nm ratio between 1.9 and 2.0, indicating pure RNA. The purified RNA was stored at -20 ºC.

RNA Concentration and Integrity:

Total RNA was then sent to the University Health Network Microarray Centre, where the quality was verified and deemed suitable for microarray hybridization. For sizing, quantification and quality of RNA, the Agilent 2100 Bioanalyzer, a microfluidics-based platform, was used. Briefly, 1 uL of the RNA sample was loaded onto each well of the chip, the sample's components were electrophoretically separated, detected by their fluorescence, and translated into electropherograms (peaks) and gel-like images (bands). An RNA ladder (a mixture of RNA of known concentration) was run in conjunction with the samples to calculate the concentration of the RNA in the sample. Furthermore, an RNA Integrity Number (RIN) score was generated on the Agilent software. For the microarray analysis, the RNA quality for all of the samples had a RIN score ≥7.

RNA Amplification:

In order to amplify the RNA for array analysis, the Illumina TotalPrep RNA Amplification Kit was used. For synthesis of first strand cDNA, the RNA sample (200 ng)

was brought up to a maximum volume of 11 µL with nuclease-free water. 9 µL of Reverse Transcription Master Mix (1 µL of T7 Oligo(dT) primer, 2 µL of 10X first strand buffer, 4 µL of dNTP mix, 1 µL of RNase inhibitor, 1 µL of ArrayScript) was added to the RNA sample and the solution was mixed thoroughly. The sample was centrifuged briefly and was then placed in the thermal cycler, where the sample was incubated for 2 hours at 42 ºC. After incubation, the sample was centrifuged briefly and placed on ice.

In order for the single-stranded cDNA to be converted into a double-stranded DNA (dsDNA) template for transcription, 80 µL of Second Strand Master Mix (63 µL of nuclease-free water, 10 µL of 10X second strand buffer, 4 µL of dNTP mix, 2 µL of DNA polymerase, 1 µL of RNase H) was added to the sample, and the solution was mixed thoroughly and centrifuged briefly. The sample was then placed in a 16 ºC thermal cycler for 2 hours. The temperature was kept at exactly 16 ºC in order to avoid compromising cRNA yield. Following the 2 hour incubation period, the sample was placed on ice.

The next step involved cDNA purification, in order to prevent the inhibition of in vitro transcription. 250 µL of cDNA Binding Buffer was added to the sample, and mixed thoroughly. The cDNA sample/cDNA Binding Buffer was then pipetted onto the centre of a cDNA Filter Cartridge. The sample was centrifuged for 1 minute at 10,000 x g, or until the mixture was through the filter. The flow-through was discarded. 500 µL of Wash Buffer was added to the cDNA Filter Cartridge, and this was centrifuged for 1 minute at 10,000 x g, or until the Wash Buffer was through the filter. The flow-through was discarded and the cDNA Filter Cartridge was centrifuged for an additional minute to remove any remaining Wash Buffer. The cDNA was then transferred to a cDNA elution tube. 20 µL of preheated nuclease-free water (55 ºC) was added to the centre of the filter in the cDNA Filter Cartridge,

and this was left at room temperature for 2 minutes and centrifuged for one minute at 10,000 x g. At this point, the double-stranded cDNA appeared in the eluate (17.5 µL). 17.5 µL of the purified cDNA sample was transferred to a PCR tube, and 7.5 µL of IVT Master Mix (2.5 µL of T7 10x reaction buffer, 2.5 µL of T7 enzyme mix, 2.5 µL of biotin-NTP mix) was added and the solution was mixed thoroughly and centrifuged briefly. The tube was then placed in the thermocycler and was incubated for 14 hours at 37 ºC. The reaction was stopped by adding 75 µL of nuclease-free water to the cRNA sample, bringing the final volume to 100 µL. The solution was then mixed thoroughly.

For cRNA purification, 350 µL of cRNA binding buffer was added to the cRNA sample, immediately followed by 250 µL of ACS reagent grade 100% ethanol. The sample was mixed by pipetting up and down. The sample was pipetted onto the centre of the filter in the cRNA filter cartridge, and centrifuged for 1 minute at 10,000 x g. The flow-through was discarded. 650 µL of wash buffer was then added to the cRNA filter cartridge, and this was centrifuged for 1 minute at 10,000 x g. The flow-through was discarded. The cRNA filter cartridge was spun again for another 1 minute in order to remove trace amounts of wash buffer. The filter cartridge was transferred to a fresh cRNA collection tube and 200 µL of nuclease-free water (preheated to 55 ºC) was added to the centre of the filter. The sample was then incubated in a 55 ºC heat block for 2 minutes, and the sample was centrifuged for 1.5 minutes at 10,000 x g. The concentration for each of the cRNA was measured on Nanodrop and the Agilent bioanalyzer was then run to determine cRNA size. 1.5 µg of cRNA was used for Illumina's direct hybridization onto the Mouse Whole genome-6 arrays.

Hybridization Array:

The Illumina WG6 V2R2 mouse array was used. This technology uses 3 μm beads that randomly assemble in a silica slide substrate. A specific oligonucleotide sequence representing a certain gene is assigned to each bead; however, there may be multiple oligonucleotide sequences that represent one particular gene throughout the array. Thus, a total of 45821 probes representing genes are represented on the array.

The biotin-modified RNA (cRNA) was hybridized to the BeadChip array for 16-20 hours at 58 ºC. Gaskets separate each array; therefore, multiple samples can be hybridized to one slide for higher throughput and reduced sample-to-sample variability. Following hybridization, the array was washed (500 mL of 1X High Temp Wash Buffer) to remove any unhybridized RNA and non-specific RNA, and the array was then stained with streptavidin-Cy3. After washing and staining, BeadChips were immediately scanned on the iScan reader, a laser based imaging system with 2 lasers (red: Cy5 and green: Cy3) for detecting fluorescence information on BeadChips. Although the iScan can scan both red and green intensities, the Illumina array is single colour therefore scans with the green laser.

5.2.6 Western Blot Analysis

Detection of several major urinary protein (MUP) protein isoforms were determined by Western blot analysis. Liver tissue was homogenized in RIPA solution, pH 8.0 (1% Triton X-100, 0.1% SDS, 150 nM NaCl, 20 mM Tris HCl, 0.5% deoxycholate) containing 2 mg/mL iodoacetamide, 2 μL/mL pestatin/leupeptin and 5 μL/mL PMSF. Protein concentrations were assayed by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Samples containing 100 ng of cellular protein were separated on an SDS-PAGE and electrically transferred to a nitrocellulose membrane (0.45 μM) in Trans-Blot® Transfer Medium (Bio-Rad Laboratories,

Hercules, CA). The membrane was incubated overnight at 4ºC with primary antibody (Santa Cruz Biotechnology Inc) diluted with a blocking buffer of 5% skim milk in PBS and 0.1% Tween 20. The membrane was then incubated with secondary antibody coupled with horse radish peroxidise (HRP) for 1 hour at room temperature. Proteins were visualized with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Pistcataway, NJ).

Table 5.1 SDS-PAGE gel and antibodies used for Western blotting assay

% of SDS-PAGE Gel	Primary antibody	Secondary antibody
10	Mouse monoclonal, 1:100	Anti-mouse HRP, 1:3000

*HRP=horse radish peroxidase

5.2.7 Statistical Analysis

For the microarray analysis, data were checked for overall quality using R (version 2.10.0) with the Bioconductor framework and the LUMI package. Data were imported in Genespring (version 11.0.1) for analysis, and data were normalized using a standard (for Illumina arrays) variance normalization followed by a "per probe" median centred normalization. All data were log2-transformed for analysis and visualization.

Data were initially filtered in order to remove any confounding effects that probes which show no signal may have on subsequent analysis. Only probes in the upper 80th percentile of the distribution of intensities in 100% of any of the 1 of 4 diet groups were not removed following filtering. After filtering, the final set contained 29992 probes.

Due to an intensity issue with samples on one slide, the samples on this slide (2 samples from the 5 mg folic acid/kg diet group and 1 sample from the 25 mg folic acid/kg

diet group) were considered a confounding variable and excluded from the analysis. Therefore, the remaining samples were analyzed: 2 samples from the 2 mg folic acid/kg diet group, 2 samples from the 8 mg folic acid/kg diet group and 1 sample from the 25 mg folic acid/kg diet group (total sample size=5). These samples were analyzed as follows:

- The 2 mg folic acid/kg diet group (control) was compared with the 8 mg folic acid/kg diet group
- The 2 mg folic acid/kg diet group (control) was compared with the 8 and 25 mg folic acid/kg diet groups combined (treatment), using an uncorrected t-test and without using a t-test (only using fold change)
- The 2 mg folic acid/kg diet group(control) was compared with the 25 mg folic acid/kg diet group, using only fold change
- The 8 mg folic acid/kg diet group was compared with the 25 mg folic acid/kg group

For each comparison, a t-test using a false discovery rate Benjamini and Hochberg multiple testing correction threshold of $p<0.05$ was initially performed. If no differences in gene expression were observed, an uncorrected t-test was then performed. If gene expression did not differ among the different diet groups following an uncorrected t-test, only fold change was examined. A corrected hypergeometric test was also performed to examine enriched Gene Ontology categories.

5.3 Results

5.3.1 Liver Folate Concentrations

Table 5.2 lists the liver folate concentrations of male offspring at 3 weeks of age chosen for the microarray analysis, and the mean folate concentration of offspring in each maternal dietary group.

Table 5.2 Liver folate concentrations (μg/g tissue) of male offspring at 3 weeks of age chosen for the microarray analysis and mean liver folate concentration (μg/g tissue) of offspring at 3 weeks of age from each maternal diet group

Sample (Maternal Diet Group)	Liver Folate Concentrations (μg/g tissue) of Male Offspring at 3 Weeks	Mean Liver Folate Concentration (μg/g tissue) of Offspring from Each Maternal Diet Group at 3 Weeks
1 (2 mg folic acid/kg diet)	4.8	4.3
2 (2 mg folic acid/kg diet)	3.1	
3 (5 mg folic acid/kg diet)*	5.2	5.7
4 (5 mg folic acid/kg diet)*	5.1	
5 (8 mg folic acid/kg diet)	8.2	7.6
6 (8 mg folic acid/kg diet)	7.1	
7 (25 mg folic acid/kg diet)*	7.6	8.8
8 (25 mg folic acid/kg diet)	10.4	

*Samples were excluded from the analysis due to an issue with the slide intensity

5.3.2 Microarray Gene Expression Analysis

No differences in gene expression were observed between the 2 mg folic acid/kg diet (control) and 8 mg folic acid/kg diet groups. Furthermore, no differences in gene expression were observed between the 8 mg folic acid/kg diet and 25 mg folic acid/kg diet groups.

Upon comparing the control and treatment (8 and 25 mg folic acid/kg diet) groups, using an uncorrected t-test, we found 477 probes representing genes that were significantly altered. However, the expression of only 2 genes, secreted phosphoprotein 1 (*Spp1*) and zinc finger and BTB domain containing 16 (*Zbtb16*), appeared to be significantly differentially expressed (*Spp1* was downregulated and *Zbtb16* was upregulated) with at least a 2-fold difference between offspring from the treatment and control groups. We then examined only fold change, without using a t-test, and found 93 probes representing genes that were differentially expressed (79 downregulated and 14 upregulated) in offspring from the maternal treatment group compared to offspring from the maternal control diet group (≥2-fold) **(Table 5.3)**. Gene ontology analysis revealed that the differentially expressed genes in offspring are involved in numerous biological pathways **(Table 5.4)**.

Table 5.3 Probes representing genes differentially expressed in offspring from the maternal treatment (8 and 25 mg folic acid/kg diet) group compared to offspring from the maternal control (2 mg folic acid/kg diet) group

Gene Symbol	Official Gene Name	Direction of Change in Gene Expression	Fold-Change
C730007P19Rik	RIKEN cDNA C730007P19 gene	↓	4.157865
Dmbt1	deleted in malignant brain tumors 1	↓	3.4029615
Slc25a25	solute carrier family 25	↓	3.3267543
Spp1	secreted phosphoprotein 1	↓	3.2384055
Gpx1	glutathione peroxidase 1	↓	3.1339684
Trf	transferrin	↓	3.0496838
Sult2a6	sulfotransferase family 2A, dehydroepiandrosterone (DHEA)-preferring, member 6	↓	3.007757
Hp	haptoglobin	↓	2.987949
Hp	haptoglobin	↓	2.9484293
Adh1	alcohol dehydrogenase 1	↓	2.8458264
Acta2	actin, alpha 2, smooth muscle, aorta	↓	2.8327608
Myh4	myosin, heavy polypeptide 4, skeletal muscle	↑	2.791003
Tff3	trefoil factor 3, intestinal	↓	2.7826564
Rnase4	ribonuclease, RNase A family 4	↓	2.7193403
Gnmt	glycine N-methyltransferase	↓	2.6818888
Spink3	serine peptidase inhibitor, Kazal type 3	↓	2.6263433
Apob	apolipoprotein B	↓	2.611458
Mup3	major urinary protein 3	↑	2.5989785
Ahsg	alpha-2-HS-glycoprotein	↓	2.5487747
Gm6484	predicted gene 6484	↓	2.5084577
Itih4	inter alpha-trypsin inhibitor, heavy chain 4	↓	2.5005949
Cish	cytokine inducible SH2-containing protein	↓	2.4973605
Serpina1e	serine (or cysteine) peptidase inhibitor, clade A, member 1E	↑	2.4752014
Sepp1	selenoprotein P, plasma, 1	↓	2.4737635
Cps1	carbamoyl-phosphate synthetase 1	↓	2.4695735
LOC673589		↓	2.4688766
Hamp	hepcidin antimicrobial peptide	↓	2.4687772
Pnpla3	patatin-like phospholipase domain containing 3	↓	2.4631224
Mup2	major urinary protein 2	↑	2.4418879
Gstt3	glutathione S-transferase,	↓	2.4389374

	theta 3		
Mup1	major urinary protein 1	↑	2.4384458
Errfi1	ERBB receptor feedback inhibitor 1	↓	2.426976
Rps6	ribosomal protein S6	↓	2.4245875
Kng2	kininogen 2	↓	2.4077525
Serpind1	serine (or cysteine) peptidase inhibitor, clade D, member 1	↓	2.3806624
Rps2	ribosomal protein S2	↓	2.3749504
Amy2-2	amylase 2-2, pancreatic	↓	2.364268
Gchfr	GTP cyclohydrolase I feedback regulator	↓	2.363034
Cps1	carbamoyl-phosphate synthetase 1	↓	2.3610566
Tff2	trefoil factor 2 (spasmolytic protein 1)	↓	2.3582907
Hrsp12	heat-responsive protein 12	↓	2.351372
Zbtb16	zinc finger and BTB domain containing 16**l**	↑	2.3473103
Mup6	major urinary protein 6	↑	2.3455088
Hpx	hemopexin	↓	2.3189836
H19	H19 fetal liver mRNA	↓	2.3168874
Mup2	major urinary protein 2	↑	2.3162546
Cyp2a5	cytochrome P450, family 2, subfamily a, polypeptide 5	↓	2.3118684
Gls2	glutaminase 2 (liver, mitochondrial)	↓	2.3112001
Serpina3m	serine (or cysteine) peptidase inhibitor, clade A, member 3M	↓	2.302695
Apom	apolipoprotein M	↓	2.288245
Cyp2c70	cytochrome P450, family 2, subfamily c, polypeptide 70	↓	2.283296
Rnase4	ribonuclease, RNase A family 4	↓	2.280903
9030024J15Rik	RIKEN cDNA 9030024J15 gene	↑	2.1990747
Gls2	glutaminase 2 (liver, mitochondrial)	↓	2.1907306
Aldh1l1	aldehyde dehydrogenase 1 family, member L1	↓	2.1875079
LOC386067		↓	2.1862917
Agxt	alanine-glyoxylate aminotransferase	↓	2.1577098
Mybpc2	myosin binding protein C, fast-type	↑	2.1524754
C730009D12		↓	2.1518865
Hamp2	hepcidin antimicrobial peptide 2	↓	2.1464007
Alas1	aminolevulinic acid synthase 1	↓	2.1344855

Es1	esterase 1	↓	2.131189
Rarres2	retinoic acid receptor responder (tazarotene induced) 2	↓	2.1299
Ang	angiogenin, ribonuclease, RNase A family, 5	↓	2.127283
Dhcr24	24-dehydrocholesterol reductase	↓	2.1159708
Angptl4	angiopoietin-like 4	↑	2.1141975
LOC234882	peroxiredoxin pseudogene 2	↓	2.1082134
Nnmt	nicotinamide N-methyltransferase	↓	2.10294
Csrp3	cysteine and glycine-rich protein 3	↑	2.0909228
Gnmt	glycine N-methyltransferase	↓	2.0863216
Gstp1	glutathione S-transferase, pi 1	↓	2.0747774
Plg	plasminogen	↓	2.0740826
Actg2	actin, gamma 2, smooth muscle, enteric	↓	2.0730474
D1Ertd471e	DNA segment, Chr 1, ERATO Doi 471, expressed	↓	2.0680013
LOC100047937		↓	2.0666313
Tfrc	transferrin receptor	↓	2.0570374
H47	histocompatibility 47	↓	2.0563748
Scp2	sterol carrier protein 2, liver	↓	2.0535948
Ctgf	connective tissue growth factor	↓	2.0488243
Spcs1	signal peptidase complex subunit 1 homolog (S. cerevisiae)	↓	2.047769
Agt	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	↓	2.0462236
Mup2	major urinary protein 2	↑	2.0408385
G6pc	glucose-6-phosphatase, catalytic subunit	↓	2.0370827
Cldn2	claudin 2	↓	2.0342345
Ccl9	chemokine (C-C motif) ligand 9	↓	2.0283618
H19	H19 fetal liver mRNA	↓	2.0269318
Vkorc1	vitamin K epoxide reductase complex, subunit 1	↓	2.0184245
Vkorc1	vitamin K epoxide reductase complex, subunit 1	↓	2.0155742
LOC224163		↓	2.0122373
Serpinf2	serine (or cysteine) peptidase inhibitor, clade F, member 2	↓	2.0121908

Cyp2d26	cytochrome P450, family 2, subfamily d, polypeptide 26	↓	2.0115285
Actn3	actinin alpha 3	↑	2.0061402
4930529C04Rik	RIKEN cDNA 4930529C04 gene	↓	2.0004861

Table 5.4 Gene ontology results (in order of the greatest to least proportion of genes altered) in offspring from the maternal treatment (8 and 25 mg folic acid/kg diet) group compared to offspring from the maternal control (2 mg folic acid/kg diet) group

Biological Pathway
defense response to fungus
polysome
endothelial cell development
iron ion homeostasis
positive regulation of lipid metabolic process
transition metal ion homeostasis
selenium binding
unspecific monooxygenase activity
triacylglycerol metabolic process
oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, reduced flavin or flavoprotein as one donor, and incorporation of one atom of oxygen
acylglycerol metabolic process
glycerolipid metabolic process
neutral lipid metabolic process
glycerol ether metabolic process
muscle contraction
serine-type endopeptidase inhibitor activity
di-, tri-valent inorganic cation homeostasis
heme binding
tetrapyrrole binding
endopeptidase inhibitor activity
microsome
protease inhibitor activity
vesicular fraction
metal ion homeostasis
regulation of lipid metabolic process
cation homeostasis

Upon comparing the control and 25 mg folic acid/kg diet groups, using an uncorrected t-test, no genes were significantly altered with at least a 2-fold difference between offspring from the treatment and control groups. Using only fold change as a viable analysis choice for this set of samples, and increasing the fold-change cut-off to ≥5 in order to restrict the results and to limit false positive results, we found 59 probes representing genes (53 downregulated and 6 upregulated) that were found to be differentially expressed in offspring from the maternal 25 mg/kg diet group compared to offspring from the maternal control diet group (≥5-fold) **(Table 5.5)**. Gene ontology analysis revealed that the differentially expressed genes are involved in numerous biological pathways **(Table 5.6)**.

Table 5.5 Probes representing genes differentially expressed in offspring from the maternal 25 mg/kg diet group compared to offspring from the maternal control (2 mg/kg diet) group

Gene Symbol	Gene Name	Direction of Change in Gene Expression	Fold-Change
Mup2	major urinary protein 2	↑	31.429869
C730007P19Rik	RIKEN cDNA C730007P19 gene	↓	29.37296
Mup2	major urinary protein 2	↑	27.394802
Mup1	major urinary protein 1	↑	20.037157
H19	H19 fetal liver mRNA	↓	18.449064
Mup6	major urinary protein 6	↑	14.684972
Mup2	major urinary protein 2	↑	14.434887
H19	H19 fetal liver mRNA	↓	11.656083
Mup3	major urinary protein 3	↑	11.183573
Rps2	ribosomal protein S2	↓	9.078135
Trf	transferrin	↓	8.564537
Gpx1	glutathione peroxidase 1	↓	8.512067
Acta2	actin, alpha 2, smooth muscle, aorta	↓	8.180789
Rps6	ribosomal protein S6	↓	7.6747146
Hp	haptoglobin	↓	7.29252
Adh1	alcohol dehydrogenase 1 (class I)	↓	7.2907557
Rnase4	ribonuclease, RNase A family 4	↓	7.0093927
Hp	haptoglobin	↓	6.9713097
Sparc	secreted acidic cysteine rich glycoprotein	↓	6.6086636
Cps1	carbamoyl-phosphate synthetase 1	↓	6.5601788
Fmo3	flavin containing monooxygenase 3	↓	6.405058
Rpl9	ribosomal protein L9	↓	6.34029
LOC382885		↓	6.135106
Rnase4	ribonuclease, RNase A family 4	↓	6.132919
Apob	apolipoprotein B	↓	6.1166124
Rps4x	ribosomal protein S4, X-linked	↓	6.096184
EG666577	predicted pseudogene 8172	↓	6.039172
Tuba1b	tubulin, alpha 1B	↓	5.9712853
Es1	esterase 1	↓	5.884281
Spcs1	signal peptidase complex subunit 1 homolog	↓	5.8346777
Adssl1	adenylosuccinate synthetase like 1	↓	5.8309855
Gnmt	glycine N-methyltransferase	↓	5.746985
Cyp2a5	cytochrome P450, family 2, subfamily a, polypeptide 5	↓	5.7444873
Tnnc2	troponin C2, fast	↓	5.68578
Cyp2c70	cytochrome P450, family	↓	5.595373

	2, subfamily c, polypeptide 70		
Serpina3m	serine (or cysteine) peptidase inhibitor, clade A, member 3M	↓	5.495219
Hamp	hepcidin antimicrobial peptide	↓	5.4843707
Lpl	lipoprotein lipase	↓	5.4746
Cyp7a1	cytochrome P450, family 7, subfamily a, polypeptide 1	↓	5.3275743
Cps1	carbamoyl-phosphate synthetase 1	↓	5.3226867
Rps7	ribosomal protein S7	↓	5.310927
LOC673589		↓	5.2796426
Gchfr	GTP cyclohydrolase I feedback regulator	↓	5.2762756
Itih4	inter alpha-trypsin inhibitor, heavy chain 4	↓	5.2723866
Mylpf	myosin light chain, phosphorylatable, fast skeletal muscle	↓	5.269612
Acta1	actin, alpha 1, skeletal muscle	↓	5.2591877
Sult2a	sulfotransferase family 2A, dehydroepiandrosterone (DHEA)-preferring, member 6	↓	5.24984
LOC386067		↓	5.2444534
LOC622655		↓	5.243467
Ppia	peptidylprolyl isomerase A	↓	5.226975
Serpind1	serine (or cysteine) peptidase inhibitor, clade D, member 1	↓	5.216999
Atp2a1	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	↓	5.214583
Rarres2	retinoic acid receptor responder (tazarotene induced) 2	↓	5.214096
Amy2-2	amylase 2-2, pancreatic	↓	5.2081127
Tnni2	troponin I, skeletal, fast 2	↓	5.130019
LOC238836		↓	5.079871
Uqcrh	ubiquinol-cytochrome c reductase hinge protein	↓	5.0767593
Kng2	kininogen 2	↓	5.0662675
Rpl18	ribosomal protein L18	↓	5.0477366

Table 5.6 Gene ontology results (in order of the greatest to least proportion of genes altered) in offspring from the maternal 25 mg/kg diet group compared to offspring from the maternal 2 mg/kg (control) diet group

Biological Pathway
triacylglycerol catabolic process
glycerol ether catabolic process
neutral lipid catabolic process
acylglycerol catabolic process
glycerolipid catabolic process
polysome
skeletal muscle fiber development
muscle fiber development
triacylglycerol metabolic process
acylglycerol metabolic process
glycerolipid metabolic process
neutral lipid metabolic process
glycerol ether metabolic process
cellular lipid catabolic process
structural constituent of ribosome
microsome
vesicular fraction
ribosome
monooxygenase activity
translation
structural molecule activity
ribonucleoprotein complex
macromolecule biosynthetic process
endoplasmic reticulum
cellular biosynthetic process
cytoplasmic part

5.3.3 Protein Expression of Major Urinary Protein

MUP protein level was increased in offspring from mothers that consumed the 25 mg folic acid/kg diet (**Figure 5.2**).

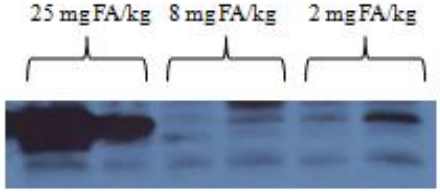

Figure 5.2 Western blot analysis for the expression of major urinary protein (MUP) in male offspring 3 weeks of age from the 2, 8, and 25 mg folic acid/kg diet maternal diet groups

5.4 Discussion

Several *in vitro*, animal, and human studies suggest that folate supplementation is capable of altering the expression of a variety of genes [25-29]. However, few studies, to date, have examined whether exposure to high levels of folic acid alone *in utero* and/or during the early postnatal period can modulate gene expression in the offspring. Thus, the primary aim of the present study was to investigate the effect of isolated maternal folic acid supplementation *in utero* and during lactation on global gene expression patterns in the liver of offspring, using supplemental levels of folic acid equivalent to 2X, 4.5X, and 12.5X the RDA in humans. These levels reflect the amount of folate and folic acid that many women of childbearing age are consuming in the post-fortification era. To our knowledge, the results from the present study demonstrate for the first time that isolated maternal folic acid supplementation provided *in utero* and during lactation can alter the expression of numerous genes in the liver of juvenile male offspring.

Due to a conflict with the intensity on one of the slides containing several samples, male offspring from the maternal 5 mg/kg diet group were not included in the analysis. We first compared the expression of genes in male offspring at 3 weeks of age from the 8 and 25 mg folic acid/kg diet groups combined (treatment) with offspring from the maternal control diet group. We found that 93 probes representing genes were differentially expressed (≥2-fold) in male offspring at 3 weeks of age from the treatment group compared to the control group (79 downregulated and 14 upregulated). We also compared the expression of genes in offspring at 3 weeks of age from the maternal 25 mg folic acid/kg diet group with offspring from the maternal control diet group, and found that 59 probes representing genes were differentially expressed (≥5-fold) in offspring at 3 weeks of age from the maternal 25 mg

folic acid/kg diet group compared to offspring from the control group (53 downregulated and 6 upregulated). These differentially expressed genes are involved in numerous biological processes, including lipid metabolism, coagulation, and iron transport and homeostasis.

Interestingly, the majority of the genes differentially expressed in the present study appeared to be downregulated following maternal folic acid supplementation. However, supplementation with folic acid to the maternal diet appeared to substantially increase the expression of several *Mup* genes, including *Mup1, Mup2, Mup3*, and *Mup6*. In addition, we performed Western blot analysis for the detection of several MUP protein isoforms, and protein levels of MUP appeared to be significantly greater in offspring from the maternal 25 mg folic acid/kg diet group compared to offspring from the control group.

Mup1, Mup2, Mup3, and *Mup6* genes belong to a multigene family encoding proteins that are part of a large family of low-molecular weight ligand-binding lipocalins [284]. Members of the lipocalin superfamily share a common tertiary structure, consisting of a cup-shaped hydrophobic ligand binding pocket surrounded by an eight-stranded β-barrel [285]. Mouse MUPs are synthesized in the liver, secreted through the kidneys, and excreted in urine [286]. It has been estimated that between 15 and 25 genes and pseudogenes exist in the mouse, which are clustered in a single locus on chromosome 4. The expression of numerous *Mup* genes has been shown in previous studies to be regulated by testosterone, thyroxine, and growth hormone, and male adult mice appear to express much higher levels than females or juveniles [286]. In addition, it has recently been shown that the expression of *Mup1* in particular is regulated by nutrient signals, as fasting and caloric restriction have been shown to dramatically reduce mouse liver *Mup1* expression, and *Mup1* expression appeared to be reversed following caloric restriction [284].

MUPs have been suggested to act as pheromone carrier proteins in mice, transporting volatile pheromones into the mucus filled pheromone detection organ. In addition, MUPs have been demonstrated to function as pheromone stabilizers in the environment, providing a slow release mechanism that extends the effective potency of these volatile molecules in male urine scent marks, and have recently been suggested to be a source of genetically encoded pheromones themselves [286].

It has recently been suggested that MUPs may also play an important role in glucose and lipid metabolism. Hui *et al*. recently showed that in a genetically inherited diabetic and obese mouse model, the expression of *Mup1* was markedly decreased (~30-fold) in liver compared to their lean littermates [285]. In mice with genetic- or dietary-induced type 2 diabetes, liver-specific overexpression of MUP1 substantially reduced hyperglycemia and glucose intolerance [287]. In addition, chronic administration of purified recombinant MUP1 proteins ameliorated hyperglycemia and improved glucose intolerance in mice with genetic-induced type 2 diabetes [285]. MUP1 therapy has also been shown to improve systemic insulin sensitivity in diabetic mice [285, 287]. It has been suggested that MUP1 may reduce blood glucose levels by suppressing the hepatic gluconeogenic program. Zhou *et al*. recently demonstrated in both animals and primary hepatocyte cultures, that recombinant MUP1 inhibited the expression of phosphoenolpyruvate carboxykinase (*Pck1*) and glucose-6-phosphatase (*G6pc*), genes which code for rate-limiting enzymes in gluconeogenesis [287]. Interestingly, in the present study, *G6pc* was down-regulated in offspring from folate-supplemented mothers. Zhou *et al*. also found that MUP1 decreased hepatic glucose production independently of insulin [287]. In addition, chronic MUP1 treatment was also shown to decrease the level of plasma lipids in genetic-induced type 2 diabetic mice, and

overexpression of MUP1 was shown to result in a marked reduction in hepatic lipid levels, likely due to suppression of lipogenic genes in the liver [285, 287].

To our knowledge, no previous studies have reported a relationship between folate and MUPs. However, given MUPs suggested role in glucose and lipid metabolism, and the substantial (~30-fold) upregulation of several *Mup* genes in the offspring in response to maternal folic acid supplementation in the present study, further investigation in this area is required. In addition, more elaborate studies are required to obtain a better understanding of the relationship between MUPs in mice and in humans [288]. Although there are numerous *Mup* genes present in mice, humans have no full-length *MUP* genes and only a single *MUP* pseudogene; however, it has been suggested that MUPs in mice may have some similarity to the human lipocalin 9 protein [289].

In the present study, we also found that the imprinted *H19* gene was downregulated in the liver of offspring from mothers that consumed a diet supplemented with folic acid. Most autosomal genes are expressed from both the paternal and maternal alleles; however, imprinted genes are expressed predominantly or exclusively from either the maternal or paternal allele in a parent of origin-specific manner. Imprinted genes often occur in clusters throughout the genome, and are typically jointly regulated through an ICR or DMR [290]. The cluster containing the maternally expressed *H19* gene also contains the paternally expressed *IGF2* imprinted gene [290]. This cluster resides at 11p15.5 in humans, and is found on chromosome 7 in mice [290]. The *H19* DMR maps between *H19* and *IGF2*, containing binding sites for a zinc-finger protein CCCTC-binding factor (CTCF) [291]. The CTCF can bind to the unmethylated *H19* DMR on the maternal allele, and insulates the *IGF2* promoter region from an enhancer downstream of *H19*. Thus, the enhancer preferentially

interacts with the *H19* promoter and the untranslated *H19* RNA is expressed from the maternal allele, while *IGF2* expression is silenced. On the other hand, the *H19* DMR is methylated on the paternal allele and CTCF is unable to bind; therefore, the downstream enhancer preferentially interacts with the *IGF2* promoter, and *IGF2*, but not *H19*, is expressed from the paternal allele [291]. Both *H19* and *IGF2* genes are widely expressed during embryonic development, but postnatally downregulated in most tissues [290]. *H19* has been shown to be highly expressed in the developing embryo in mesoderm and endoderm-derived tissues, while shortly following birth, this gene is expressed only in cardiac and skeletal muscle [292].

Although imprinted genes are relatively few in number, these genes appear to play a critical role in various human diseases. In humans, the 11p15.5-imprinted domain has been linked to Beckwith-Wiedemann syndrome (BWS), a congenital disorder commonly characterized by overgrowth phenotypes in children as well as predisposition to develop embryonal tumors [293]. 5-10% of patients with BWS develop tumors, including Wilms tumor, hepatoblastomas, rhabdomysarcomas, adrenocortical carcinoma, and neuroblastomas [291]. Approximately 5% of BWS cases display gain of maternal allele methylation at the *H19* DMR, resulting in biallelic expression of *IGF2*, and silencing of *H19* expression [291].

H19 encodes a fully processed 2.3 kb non-coding, spliced, and polyadenylated RNA. At present, its function has not been clearly established. However, it has been suggested that *H19* is a tumor suppressor gene, which would provide some insight into the predisposition of BWS children to develop embryonic tumors during the earliest years of life [292]. Yoshimizu *et al*. recently showed that transfection of *H19* cDNA into G401-transformed kidney cells demonstrated loss of tumorigenicity of these cells [293]. However, *H19* has also

been recently shown to induce proliferation of breast cancer cells through the activation of the transcription factor E2F1, suggesting that the gene may have oncogenic properties [294].

Due to the unique epigenetic requirements associated with allele-specific expression, genomically imprinted genes may be particularly sensitive to epigenetic dysregulation by environmental influences during development. Mouse embryos cultured in the presence of fetal calf serum demonstrated reduced viability, reduced body weight, decreased expression of *H19* and *Igf2* and increased methylation of the *H19* ICR [295]. Lillycrop *et al*. recently showed that the imprinted genes insulin I (*Ins1*) and pleckstrin homology-like domain, family A, member 2 (*Phlda2*) were significantly altered in offspring from mothers that consumed a protein-restricted diet supplemented with folic acid compared to offspring from mothers that consumed only the protein-restricted diet [30]. In addition, in humans, *H19* loss of imprinting in patients with hyperhomocysteinaemia was ameliorated by folic acid supplementation [29]. Interestingly, Dobosy *et al*. recently observed a significant increase in both *Igf2* and *H19* expression in choline and methionine deficient prostate tissues of mice, and these expression changes appeared to be reversible [296]. Significant decreases in repressible histone modifications within the *H19* promoter, as well as *Igf2* P2 and P3 promoters were observed, while the methylation status of specific CpG islands within the *Igf2* and *H19* promoters of the prostate was not altered, suggesting that histone modification may be more susceptible to a methyl-deficient diet than DNA methylation in the short term [296]. The observed decrease in *H19* expression in the present study in response to maternal folic acid supplementation warrants further investigation into the influence of high folate exposure during the earliest stages of life on the methylation status and expression of imprinted genes, including *H19* and *Igf2*.

In the present study, we also found that several genes involved in lipid metabolism were downregulated in the present study in offspring from mothers that consumed a folate-supplemented diet, including apolipoprotein B (*Apob*), apolipoprotein M (*Apom*), and lipoprotein lipase (*Lpl*). APOB is the primary protein constituent of low-density lipoproteins (LDL) [297]. Since APOB levels are a direct measurement of potentially atherogenic particles, APOB has been suggested to be a more appropriate trait to measure risk of cardiovascular disease than LDL; however, LDL is often more clinically utilized [298]. APOM, another lipoprotein-associated protein, is mainly found in high-density lipoproteins (HDL) but is also present in chylomicrons, very-LDL and LDL [299]. Interestingly, the structure of APOM is most closely related to that of the rat MUP, as these proteins have similar lipocalin folds; however, APOM and MUP only share 19% amino acid sequence identity [300]. LPL hydrolyzes triglycerides in plasma lipoproteins to fatty acids and monoglycerides, a pre-requisite for their utilization in tissues [301].

Interestingly, a recent randomized, double-blind, placebo-controlled human intervention study by Duthie *et al*. examined the effect of folic acid supplementation (1.2 mg/day) for 12 weeks on the plasma proteome in normal healthy subjects and found that folic acid supplementation decreased (by almost 9-fold) the expression of plasma apolipoprotein A-I, the major protein component of HDL [302]. In addition, preintervention, high concentrations of circulating 5-methylTHF were associated with low apolipoprotein A-IV expression [302]. Furthermore, McNeil *et al*. recently showed that a diet deficient in folate alone or a methyl-deficient diet consumed in pregnant mothers throughout pregnancy altered lipid metabolism in the fetal liver [253].

The findings from those studies as well as the present study suggest that folic acid supplementation, including *in utero* and during lactation, may influence lipid metabolism and transport. It has been suggested that low levels of folate may pose a risk for cardiovascular disease, since inadequate intake of folate or combinations of B vitamins increase homocysteine levels, an independent biomarker for cardiovascular disease and stroke [303]. In addition, observational studies suggest that individuals with high dietary intake or plasma concentrations of folate have a decreased risk of coronary heart disease. However, a recent randomized trial found that following 7.3 years of treatment and follow-up, total cardiovascular events among high-risk women were not reduced by vitamin supplementation [304]. In addition, a recent meta-analysis found that folic acid supplementation did not reduce the risk of cardiovascular disease or all-cause mortality among participants with a history of vascular disease [305]. In another recent meta-analysis including 8 randomized trials of folic acid supplementation, however, a significant reduction in stroke was observed with folic acid supplementation among individuals with no prior history of stroke [306]. The possibility that maternal folic acid supplementation may alter lipid metabolism in the offspring, and that high doses of folic acid may play a role in cardiovascular disease, warrants further investigation into this area.

Alterations in folate status may also influence the expression of genes involved in coagulation. In the study by Duthie *et al*., low baseline SAM was associated with low kininogen 1 protein expression, and following folic acid supplementation, kininogen expression was positively associated with plasma total homocysteine and negatively with uracil misincorporation. Kininogen 1, also known as bradykinin, is involved in the regulation of blood pressure, diuresis, and coagulation [302]. In the present study, we found that

maternal folic acid supplementation downregulated the expression of kininogen 2 (*Kng2*) in male offspring from mothers who consumed a diet supplemented with folic acid, compared to offspring from mothers on the control diet. Duthie *et al*. also observed a decrease in antithrombin III protein expression following folate supplementation. Interestingly, we found that maternal folic acid supplementation downregulated serpin peptidase inhibitor, clade D (heparin cofactor) member 1 (*Serpind1*). The *Serpind1*gene codes for the protein heparin cofactor II. In humans, heparin cofactor II is a single-chain glycoprotein that is homologous to antithrombin and other members of the serpin family, which rapidly inhibit thrombin [307]. We also found that several other genes which code for proteins involved in coagulation were downregulated following maternal folic acid supplementation, including serine (or cysteine) peptidase inhibitor, clade F, member 2 *(Serpinf2)* and plasminogen (*Plg*).

Several genes involved in iron transport and homeostasis were also downregulated in offspring from mothers that consumed a diet supplemented with folic acid, including transferrin (*Trf*), transferrin receptor (*Tfrc*), hepcidin antimicrobial peptide *(Hamp)* and hepcidin antimicrobial peptide 2 (*Hamp2*). *Trf* codes for a highly conserved serum glycoprotein involved in iron transport [308]. The TRF protein has two high-affinity binding sites for Fe(III), and maintains iron in a soluble form, serves as a major vehicle for iron delivery into cells, and limits the generation of toxic radicals [309]. There are numerous mechanisms by which transferrin can deliver iron to cells, including the high affinity binding of the protein to the plasma membrane protein TRFC [310]. The TRFC is found on the surface of most cells in the body, and deficiency has been shown to lead to embryonic lethality in mice [310]. *Hamp* codes for a protein which orchestrates systemic iron fluxes and controls plasma iron levels by binding to the iron exporter ferroportin on the surface of iron-

releasing cells, triggering its degradation and hence reducing iron transfer to TRF [309]. We also found that maternal folic acid supplementation decreased the expression of haptoglobin (*Hp)*, the gene coding for an α-2 sialoglycoprotein found in plasma, which binds free haemoglobin [311]. Thus, HP prevents the release of heme iron which can cause oxidative damage. HP also plays a role in the immune system, by inhibiting lymphocyte proliferation, granulocyte chemotaxis/phagocytosis, neutrophil respiratory burst activity, prostaglandin synthesis and angiogenesis [302]. Interestingly, Duthie *et al*. found that high folate status was associated with high haptoglobin protein expression before and following intervention with folic acid [302].

The results from the present study suggest that maternal folic acid supplementation during the intrauterine and lactation periods can alter the expression of numerous genes in the offspring. In a recent genome-wide gene expression analysis, Lillycrop *et al*. showed that a maternal protein-restricted diet supplemented with a moderate degree of folic acid (5 mg folic acid/kg diet) was able to prevent some changes in gene expression in the offspring that were altered following a maternal protein-restricted diet alone; however, folic acid supplementation did not correct for other changes, and in certain cases, genes that were not differently expressed from the maternal protein-restricted diet alone demonstrated significant changes following folic acid supplementation [30]. In addition, Caldwell *et al*. found that more than 80 transcripts in the differentiation, more than 60 transcripts in the adhesion, apoptosis, and cell cycle, and more than 50 transcripts in the cytoskeleton pathways, were altered in the fetal heart exposed to supplemental levels of folate (8 mg folic acid/kg diet) *in utero* [254].

Since folate is directly involved in the synthesis of SAM, and folate supplementation has been shown to alter both genomic- and gene-specific DNA methylation, one plausible mechanism by which maternal folic acid supplementation may induce gene expression changes in the offspring is through DNA methylation of CpG promoter islands. Other epigenetic mechanisms, including histone modifications, may also play a role. Inadequate prenatal nutrition has been shown to alter transcriptional activity in the offspring through changes in the epigenetic regulation of both DNA methylation and histone modifications [31, 312, 313].

The observed changes in gene expression may not all have functional consequences; however, altered gene expression could induce phenotypic changes in the offspring, many of which may not manifest until later in life. To date, several recent human studies have suggested that folic acid supplementation may be associated with potential adverse effects in certain segments of the population, such as increased NK cytotoxicity and an increased risk of certain cancers [50, 103]. In offspring from mothers with a high folate status, folic acid supplementation has been suggested to increase the risk of obesity, insulin resistance, and asthma [15, 16, 139]. Thus, it is critically important for future studies to examine whether changes in gene expression as a result of maternal folic acid supplementation persist into adulthood, and whether these changes are associated with phenotypic changes in the offspring which may ultimately influence health outcomes in the offspring.

5.5 Conclusion

Notwithstanding the limitations of the present study, our data suggest that high levels of maternal folic acid supplementation during pregnancy and lactation are capable of altering

the expression of numerous genes in the liver of juvenile offspring, including numerous *MUP* genes, the imprinted *H19* gene, and genes involved in numerous biological processes including lipid metabolism, coagulation, and iron transport and homeostasis. Future studies are required to determine the effects of maternal folic acid supplementation on gene expression in female offspring, whether these effects are tissue-specific, whether these effects persist into adulthood, and whether any phenotypic changes and functional ramifications are associated with alterations in gene expression.

Chapter 6: GENERAL DISCUSSION, LIMITATIONS AND FUTURE DIRECTIONS

6.1 Overall Summary

The results from the present study demonstrate that increasing doses (2.5x, 4x, and 12.5x BDR) of folic acid supplementation provided *in utero* and during lactation can induce global DNA hypomethylation in the liver of juvenile offspring, which persists into adulthood, and that high levels of maternal folic acid supplementation (4x and 12.5x BDR) provided *in utero* and during lactation can modulate the expression of numerous genes in the liver of male juvenile offspring.

6.2 General Discussion

The relationship between a mother's folate status during pregnancy and/or during lactation and the health of her offspring is becoming a topic of growing interest. In addition to the protective effect of folate against major developmental abnormalities including NTDs, as well as the role of folate in the treatment of anemia, several recent studies suggest that a high maternal folate status may be associated with a reduction in several cancers in the offspring [131, 133-135]. However, emerging evidence suggests that a high maternal folate status may also be associated with various undesirable health outcomes in the offspring [15, 139]. In rats, male offspring from mothers that consumed a low-protein diet supplemented with folic acid during pregnancy demonstrated a decrease in growth rate after 7 months, an increase in blood glucose and corticosterone concentrations, and 40% lower brain concentrations of docosahexaenoic acid compared to offspring from mothers on the control

diet [136, 137]. In addition, a recent study from our laboratory in the dimethylbenzanthracene (DMBA) rodent model found that high intrauterine and early postnatal exposures to folic acid increased the risk of breast cancer development in the offspring [138]. In humans, an increased risk of neuroblastoma in the offspring was associated with maternal folate supplementation [139]. The Pune Maternal Nutrition Study in India found that maternal serum folate levels in humans were positively associated with adiposity and insulin resistance in offspring at 6 years of age. The offspring of mothers deficient in vitamin B_{12} but folate replete demonstrated the greatest degree of insulin resistance [15]. Additionally, the recent Norwegian Mother and Child Cohort Study demonstrated that supplementation with folic acid during the first trimester of pregnancy was associated with increased risk of wheeze and respiratory tract infections in offspring up to 18 months of age [16].

At present, the biological mechanisms by which maternal folate status can influence the immediate and long-term health of the offspring are largely unknown. However, persistent changes in the offspring phenotype ultimately imply changes in gene expression. One plausible mechanism by which alterations in maternal folate status during pregnancy and lactation may induce changes in gene expression and subsequent phenotypic changes is through epigenetic mechanisms, including DNA methylation. Folate plays an essential role in DNA methylation, and both folate deficiency and supplementation have been shown to modulate DNA methylation. However, the epigenome may be most vulnerable during the earliest stages of mammalian development, and few studies to date have examined whether exposure to isolated folic acid during the intrauterine and lactation periods are capable of modulating DNA methylation and subsequent gene expression in the developing offspring.

Thus, we designed two studies to investigate the effect of *in utero* and early postnatal folic acid supplementation on hepatic genomic DNA methylation and global gene expression in the developing offspring. Offspring from mothers consuming diets supplemented with folic acid (5, 8, and 25 mg folic acid/kg diet) demonstrated significant increases in liver folate concentrations; therefore, this animal model provided an excellent opportunity to investigate the effect of maternal folic acid supplementation on both genomic DNA methylation and global gene expression in the liver of offspring. The findings from these studies collectively suggest that isolated maternal folic acid supplementation throughout pregnancy and lactation can induce changes in genomic DNA methylation and alter the expression of numerous genes in the offspring, which could have potential functional effects on the health of the developing offspring.

In our first study, we demonstrated that a maternal diet supplemented with both a modest (5 mg folic acid/kg diet) and high amount of folic acid (8 and 25 mg folic acid/kg diet) can induce global DNA hypomethylation in the liver of juvenile offspring, and that this effect persisted into adulthood. In our second study, we showed that high levels (8 and 25 mg folic acid/kg diet) of isolated maternal folic acid supplementation can alter the expression of numerous genes in the liver of juvenile male offspring. Genes involved in numerous biological processes, including lipid metabolism, coagulation, and iron transport and homeostasis, were found to be differentially expressed, with many of these being downregulated. In addition, numerous *Mup* genes were found to be significantly upregulated, while the imprinted gene *H19* was found to be downregulated, in the offspring following maternal folic acid supplementation.

Our goal in the present animal study was to parallel maternal folic acid intake to the human condition by using supplemental levels of folic acid that are comparable to folate intakes observed in many women of childbearing age in North America. Thus, we chose varying supplemental levels of folic acid equivalent to 2X, 4.5X, and 12.5X the RDA in humans.

The results from the present study suggest that maternal folic acid supplementation can induce global DNA hypomethylation in the liver of the offspring. Furthermore, maternal folic acid supplementation altered the expression of numerous genes in the liver of juvenile offspring, many of which were downregulated. The observed downregulation of numerous genes raises the possibility that, if CpG islands or CpG sites are present in the promoter regions of some of these genes, an increase in CpG island or CpG site methylation in the promoter regions may be responsible for this downregulation. Whether or not the decreased expression of these genes is epigenetically mediated by CpG promoter methylation needs to be determined. Interestingly, neoplastic cells are often characterized by a global decrease in DNA methylation in the gene body and an increase in CpG island methylation of critical tumor suppressor and other critical cancer-related genes.

Although the functional ramifications of maternal folic acid supplementation are unknown at present, the observed global DNA hypomethylation and altered expression of numerous genes could have significant functional consequences that could affect the health and disease of the offspring.

6.3 Limitations and Future Directions

In the present study, we chose a mouse model to examine the effects of maternal folic acid supplementation during pregnancy and lactation on DNA methylation and gene expression in the offspring. There are numerous advantages of choosing mice, including their short gestation and lifespan, relatively inexpensive cost, the high extent of control over genetic and environmental variability, and the greater homology of genes with corresponding genes in humans.

However, there are some theoretical disadvantages of the animal model chosen for the present study. Rodents often have large litter sizes and these are considered a disadvantage in the sense that pups share space within the uterus and therefore each pup develops a slightly different environment [314]. Furthermore, it has also been suggested that exposure to different levels of hormones, depending on whether the fetuses are positioned next to male or female littermates, can also influence development within litters.

It is also important to note that the timing and trajectory of developmental processes differs between rodents and humans [314]. Rodents are considered altricial species - at birth, they are relatively immature compared to humans and larger animal models including sheep and pigs.

In addition, there are several differences in folate metabolism between animals and humans. For example, Bailey *et al*. have recently demonstrated that DHFR activity in humans is very low in comparison to rats [275]. The enzyme DHFR must first reduce folic acid to DHF, and then to THF. Due to the fact humans may have a very limited ability to efficiently metabolize folic acid, particularly in high doses, whether or not the supplemental

levels of folic acid used in the present study can be directly extrapolated to intake levels in humans remains a controversial issue.

Therefore, although similarities do exist, rodent models differ in several important aspects from humans and any extrapolation of the observations from these models to human situations should be made cautiously.

Specialized wire-bottomed stainless steel cages, which are often used to minimize coprophagy, were not used in the present study due to ethical considerations and the animal care committee at our institution did not allow us to use these cages. However, evidence suggests that even these special cages cannot completely prevent coprophagy in rats and that mice do not usually exhibit coprophagy as much as rats do. Studies in our lab have shown that blood and tissue folate concentrations of rats and mice in dietary folate deficiency and supplementation experiments without specific measure to prevent coprophagy were similar to those from experiments using these special cages. In addition, succinylsulfathiazole was not used in the present study to inhibit the *de novo* synthesis of folate from intestinal microflora. Folate synthesized by intestinal microflora has been shown to be incorporated into tissue folate of the host in rodents [34]. Therefore, there is a possibility that mice in the present study were exposed to more folate than the amount present in the experimental diets. Future studies should consider these issues when determining the quantity of folic acid to be added to the diets.

In addition, although beyond our control, some of the mothers may have consumed their assigned diet longer than others, as the length of time it took to become pregnant varied for each mother. Furthermore, we did not monitor food intake throughout the study; therefore, there remains the possibility that some of the mothers and offspring may have

consumed more food over the study compared to others, which could have confounded the results.

Another limitation may have been the control diet chosen for this study. For rodents, the 2 mg folic/kg diet has generally been accepted as the basal dietary requirement for rodents [315]. However, the estimated nutrient requirement set by the National Research Council is 0.5 mg folic acid/kg. Therefore, the control diet used in the present study may have been more than the requirement for mice. This may explain why there were no differences observed in homocysteine concentrations among the different maternal diet groups. Perhaps if the folic acid amount in the control diet was slightly lower, a homocysteine-lowering effect would have been observed with maternal folic acid supplementation.

Due to the cost of microarray experiments, we only used 2 samples per diet group for our microarray experiment. Furthermore, due to an intensity issue on one of the slides, we had to exclude 3 additional samples from the final analysis. Thus, one of the major limitations of our microarray study was the small sample size used, and data from data from this study should be interpreted with caution.

The results from the present study suggest that maternal folic acid supplementation provided during pregnancy and lactation can induce global DNA hypomethylation in juvenile offspring, and that this effect persisted into adulthood. In order to elucidate potential mechanisms by which maternal folic acid supplementation affected DNA methylation, it will be important to determine related markers of DNA methylation including SAM and SAH concentrations, as well as the activity level of DNMT. In addition, it will be critical to determine whether maternal folic acid supplementation can alter global CpG island DNA

methylation. We did not specifically interrogate CpG promoter island methylation in our study due to the time and cost consideration. Future studies using both epigenomic and candidate-gene approaches to study the effect of maternal folic acid supplementation on CpG promoter methylation are required. In addition, other epigenetic modifications, including histone methylation and post-transcriptional control by microRNA, should be examined.

The findings from our microarray study suggest that maternal folic acid supplementation provided *in utero* and during lactation can alter the expression of a variety of genes involved in numerous biological pathways in juvenile male offspring. Due to the fact our sample size was limited, it is critically important to confirm these results with real-time RT-PCR. In addition, performing further functional analysis including protein analysis, on other proteins in addition to MUP, to determine whether the observed changes in mRNA transcript levels are maintained through translation is critical.

Due to the fact only males at 3 weeks of age were examined, the effects of maternal folic acid supplementation on gene expression in female offspring should be determined. Several recent studies have shown that the effect of altering the maternal diet on the offspring may vary according to the sex of the offspring [259, 316]. In rats, a methyl-deficient diet fed during pregnancy was shown to program gender-specific changes in glucose handling by the offspring [316]. Thus, it will be critical to determine whether male and female offspring respond differently to intrauterine and early postnatal exposure to high levels of folic acid.

In addition, it will be important to determine whether any changes observed in gene expression in juvenile offspring persist into adulthood. Furthermore, since we could not include male offspring from the 5 mg folic acid/kg diet maternal diet group in the microarray

study, future studies should determine whether more moderate doses of folic acid supplementation are capable of inducing changes in gene expression. However, it is important to note that we did not observe any significant changes in gene expression between the control and 8 mg folic acid/kg diet maternal diet group; therefore, there remains the possibility that only very high levels of maternal folic acid supplementation can induce changes in gene expression in the offspring.

In the present study, we examined DNA methylation and gene expression only in the liver of the offspring. The effects of maternal folic acid supplementation on DNA methylation and gene expression patterns are most likely tissue-specific; therefore, it will be important to determine the effects of intrauterine and early postnatal exposure to folic acid on DNA methylation and gene expression in various other tissues from the offspring.

Folic acid supplementation was provided throughout the duration of pregnancy and lactation in the present study; thus, we were unable to distinguish whether the effect of folic acid supplementation on DNA methylation and gene expression was due to *in utero* or early postnatal exposure. Recently, Heijmans *et al*. found that early, but not late, gestational exposure to famine was associated with epigenetic changes [231]. Thus, future studies should distinguish between the *in utero* and lactation periods, in order to identify the specific period during which the developing offspring may be particularly susceptible to folic acid supplementation.

An adequate intake of folate throughout the periconceptional period is associated with a decreased risk in NTDs. In addition, adequate intake of folate throughout the life course has been suggested to be associated with a lower risk of diseases including cardiovascular disease and certain cancers. However, emerging evidence suggests that folic

acid supplementation may be associated with potential adverse effects in certain segments of the population. The long-term effects of maternal folic acid supplementation on the health of the offspring have not yet been clearly defined and may even have unintended consequences. Thus, it is critically important to further examine the effects of intrauterine and early postnatal exposure to folic acid on the developing offspring, in order to establish safe and effective recommendations and public health interventions for women of childbearing age.

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